SUBJECT: Risk Assessment for R-18-0001 - **NOT CBI**

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SUMMARY

The Agency received a TSCA Experimental Release Application (TERA) from Arizona State University (ASU) on behalf of the Producing Algae for Co-products and Energy (PACE) Consortium for one intergeneric strain of the freshwater green alga *Chlorella sorokiniana.* The submission strain for this risk assessment is *C. sorokiniana* PACE_Cs1412_SNRK2 (from now on known as PACE_Cs1412_SNRK2). The submitter plans to field test this intergeneric algal strain in open raceway miniponds at the Arizona Center for Algae Technology and Innovation (AzCATI) site in Mesa, AZ.

The recipient alga strain is *Chlorella sorokiniana* DOE1412. The submission strain, PACE_Cs1412_SNRK2, contains an intergeneric algal gene that encodes the sucrose non-fermenting related kinase 2 (SNRK2). The SNRK2 group of enzymes are implicated in both direct and indirect abscisic acid (ABA) response pathways dealing with environmental stress-signaling in plants, and was shown to confer increased sucrose synthesis, starch synthesis, and leaf growth when overexpressed in *Arabidopsis*.

The submitters expected the introduction and overexpression of *SNRK2* would improve starch accumulation and growth in *Chlorella* cells. Preliminary data in the TERA submission (R-18-0001) indicated that *C. sorokiniana* DOE1412 expressing the *SNRK2* gene had improved photosynthetic efficiency and growth compared to the wild type strain.

There is low risk of injury to human health and the environment associated with the small-scale field testing of the intergeneric alga strain PACE_Cs1412_SNRK2. The submission strain does not present concerns for pathogenicity or toxicity to humans. It does not present allergenicity concerns for normal workers or the general population. The naturally occurring *C. sorokiniana* has been grown outdoors at

the site for more than two years and other species of *Chlorella* have been grown at the site for more than 10 years with no allergenicity effects to workers. However, *Chlorella* sp. may present some concern for allergenicity to the susceptible subpopulation of atopic individuals, i.e., those with a genetic predisposition toward developing hypersensitivity reactions as one study stated that *Chlorella* is a weak allergen. If any workers are atopic, their allergy symptoms could be alleviated with the use of respirators. Although the submission strain contains a gene that confers resistance to the antibiotic zeocin, this antibiotic is not used to treat infections in humans. Therefore, there is no concern for loss of the therapeutic value of the antibiotic if the resistance gene was to be transferred to pathogens in the environment.

The small-scale field testing of the submission strain, PACE_Cs1412_SNRK2, is expected to present low risk to the environment. Since the introduced genetic material imparts enhanced growth and biomass accumulation, the submission strain could have a competitive advantage in the environment as it will consume more nutrients at a faster rate than that of the wild type recipient. However, the characteristics of enhanced growth and biomass accumulation have been extensively studied in *C. sorokiniana* and shown that similar results can be attained in the wild type by simply tuning various growth parameters.

Dispersal of the algal cells into the environment is likely since *Chlorella* is an algal species known to be routinely transmitted in air. Members of the genus *Chlorella* are known to survive in marine systems, so it is likely that the submission strain may do so as well. However, the submission strain is not expected to be invasive and outcompete other algae in the environment. Therefore, no adverse environmental effects are expected if the strain does survive in terrestrial and aquatic environments into which it is dispersed.

The horizontal gene transfer of the introduced genetic material that imparts heat and salinity tolerance, i.e., the *SNRK2* gene, to other algal species in the environment is expected to be low as *Chlorella* is not known to readily exchange genetic material horizontally. Vertical transfer of the introduced genetic material through sexual reproduction to other *Chlorella* species is also expected to be low since *Chlorella* is thought to be asexual. Although the researchers have noted that sexual reproduction of *Chlorella sorokiniana* (strain UTEX 1228) is possible through inducement of gametogenesis in the laboratory, sexual reproduction is not expected to occur naturally. Furthermore, the recipient strain for this submission *C. sorokiniana DOE1412* has not been reported to be capable of sexual reproduction. Thus, there is low concern for transfer of the introduced *SNRK2* gene to other algal in the environment. Even though there may be dispersal of the submission strain PACE_Cs1412_SNRK2 into the environment from the proposed small-scale field testing, there is low risk associated with these field tests since the submission strain poses low human health and ecological hazards.

Of note, last summer the Agency approved a TERA from the same submitters (R-17-0002) using a different recipient strain of *Chlorella sorokiniana* with introduced genetic material for heat and salinity tolerance.

I. INTRODUCTION

EPA received a TSCA Experimental Release Application (TERA) from Arizona State University on behalf of the Producing Algae for Co-products and Energy (PACE) Consortium to test one intergeneric eukaryotic

algal *Chlorella sorokiniana* construct in open raceway miniponds to gather data for the future development of potential commercialization strains.

The submission strain for this risk assessment is PACE_Cs1412_SNRK2, and the recipient microorganism is the green alga, *Chlorella sorokiniana* DOE1412. The introduced intergeneric DNA sequences in the final construct includes a gene that encodes the sucrose non-fermenting (snf) related kinase 2 (*SNRK2*) from the green alga *Picochlorum soloecismus* (R-18-0001). The *SNRK2* gene was synthesized in its native state (only the coding regions) without codon optimization. Expression of the introduced *SNRK2* gene enables the submission strain, PACE_Cs1412_SNRK2, to have an enhanced growth rate, photosynthetic efficiency, and biomass accumulation. In addition, PACE_Cs1412_SNRK2 also contains the *Streptoalloteichus hindustanus* Sh *ble* gene which confers resistance to the antibiotic zeocin that enables selection of transformants.

II. TAXONOMY AND CHARACTERIZATION

A. Recipient Microorganism

The recipient alga obtained from the University of Texas algae culture collection is *Chlorella sorokiniana DOE1412* which is also listed as UTEX B 3016, NAABB 1412, and NAABB 2412 (UTEX website - https://utex.org/products/utex-b-3016, accessed 09/2018). This species designation is accepted (Peñalva-Arana, 2018). According to Peñalva-Arana's Taxonomic Identification Report (2018), this strain was isolated from the field by Juergen Polle in 2013 and deposited to the City University of New York (CUNY) collection (UTEX website - https://utex.org/products/utex-b-3016, accessed 09/2018). Subsequently, the National Alliance for Advanced Biofuels and Bio-Products (NAABB) consortium, after a screening process, has made 30 of their best performing strains including DOE1412 available to the public through UTEX. These UTEX strains have been well characterized by DOE for lipid production and growth kinetics. UTEX and DOE describe the strain as a high temperature freshwater strain (cold-sensitive) with a maximum growth temperature of 42 °C.

1. The Genus *Chlorella*

As discussed in the Ecological Hazard Assessment (Nguyen, 2018b) and previously in a TERA submission (R-17-0002), the genus *Chlorella* was delineated by Beijerinck [Beyerinck] (Beijerinck, 1890). A comprehensive description of the genus *Chlorella* was first addressed by Shihira and Krauss (1965) in response to the lack of a sound taxonomic framework from which to base the identity of over 41 isolates known at the time. In 1976, Kessler identified 77 strains across 12 taxa based on physiological and biochemical properties. Since then the genus has been found to have few useful diagnostically morphological characteristics, and thus it is difficult to identify under a light microscope alone. Only through more rigorous methods (e.g., DNA analysis) can it be clearly classified as belonging to a specific species (Bock et al., 2011; Zou et al., 2016). A more robust framework, based on polyphasic taxonomic approaches, has been developed to describe well over 100 potentially different *Chlorella* species (Bock et al., 2011; Zou et al., 2016). Based on integrative or polyphasic taxonomy, a new system has been established which differs completely from the traditional artificial system of classifying *Chlorella* and its relatives based on morphology alone. Using small subunit (SSU) - and internal transcribed spacer (ITS) rDNA gene sequences and light microscopy observations, various publications have demonstrated the high level of cryptic diversity found within *Chlorella* and the polyphyletic characters between *Chlorella* and *Dictyosphaerium* which has resulted in numerous taxonomic revisions of these organisms (Zou et

al., 2016). For example, Bock et al. (2011) detected six lineages of *Dictyosphaerium*-like strains that are closely related to *Chlorella vulgaris* and described several new species. Krienitz et al. (2015) also attempted to demonstrate that *Chlorella* species have been widely misclassified when using traditional morphological classification schemes and suggested that only three 'true' spherical species belong to this genus: *Chlorella vulgaris*, *C. lobophora*, and *C. sorokiniana*. Based on biochemical and molecular data, the *Chlorella* genus was even more recently proposed to consist of five "true" *Chlorella* species (Zou et al., 2016). The number of *Chlorella* species appears to have reached ~14 with the inclusion of several former *Dictyosphaerium* strains (Bock et al., 2011), and with suggestions of still others possible (Zou et al., 2016). Regardless of the ongoing debate of the number of species, *C. vulgaris* is considered the type species of this genus (Shihira and Krauss, 1965) and *C. sorokiniana* has retained authentic species status throughout these various taxonomic revisions (Peñalva-Arana, 2018).

Members of the *Chlorella* genus are single-celled coccoid photosynthetic green microalgae. They are typically small (1-10 µm in diameter) and can be found as either singly or clustered cells in aquatic and terrestrial systems. The genus *Chlorella* is found within the Chlorellaceae clade, in the Trebouxiophyceae class, in the Chlorellales order (Bock et al., 2011; Huss et al., 2009). In the past, some *Chlorella* species have been attributed to the Chlorophyceae*,* but true *Chlorella* belong to the Trebouxiophyceae. C*hlorella sensu stricto* is now placed explicitly in the class Trebouxiophyceae. This class also contains most of the known green algal endosymbionts, living in lichens, unicellular eukaryotes, plants and animals (Blanc et al., 2010). Members of the true *Chlorella* genus are also nonmotile with a single chloroplast and a rigid chitinous cell wall, characterized by glucosamine as a major component of the cell wall (Takeda, 1991). These cells do not have mucilagineous envelopes or other cell wall ornamentation. They contain a single chloroplast with a pyrenoid. The pyrenoid is covered by a starch envelope and traversed by thylakoid membranes. Planktonic *Chlorellaceae* evolved into distinct forms, while terrestrial members exhibit morphological convergence, characteristic of the true *Chlorella* clade (Bock et al., 2011). Luo et al. (2010) state that in the traditional context, and also according to the first studies that included molecular and phylogenetic investigations, members of the genus *Chlorella* represent the archetype of a green spherical cell propagating purely by autosporulation (Huss et al., 1999). *Chlorella* has only been observed to reproduce asexually by nonmotile reproductive cells (autospores) that rupture through the mother cell. However, Blanc et al. (2010) reported that although *Chlorella* has long been assumed to be asexual, the genome of *C. variabilis* NC64A possesses genes encoding meiosis-specific proteins, and they also found homologs of the *Chlamydomonas* gametolysin proteins that promote disassembly of the gametic walls and allows for gamete cell wall fusion. Blanc et al. (2010) therefore suspected that meiosis and sexual reproduction are part of the *Chlorella* life cycle that may have been simply overlooked, like the cryptic sex later identified in other algae species.

The submitters previously reported that under strict laboratory conditions they have been able to induce the production of haploid gametes in intermediate transgenic strains, which allowed them to do backcrossing (to avoid any reversions to a wildtype genetic background) to arrive at the final strain for their last TERA submission (R-17-0002). They also noted that in a closely related strain (*C. sorokiniana* 1228), rare indications of haploids and sexual reproduction were observed by microscopy. However, backcrossing was not done for this submission strain, PACE_Cs1412_SNRK2, since *SNRK2* overexpression has been observed by the submitters (PCR/RT-PCR positive amplicons) to be conserved in the transgenic lines for two years. As a result, back-crossing was considered unnecessary in this specific case for the stability of *SNRK2* overexpression in PACE_Cs1412_SNRK2.

Members of the genus *Chlorella* are found in fresh waters, marine waters, and in edaphic environments including desert soil crusts (Treves et al., 2016). According to Hodac et al. (2016) *Chlorella* are

omnipresent in terrestrial and aquatic habitats from polar to temperate to tropical climates.

Some *Chlorella* species, including *C. sorokiniana*, are highly temperature tolerant (T_{max} 45°C) (Sayre et al., 2015). This well-researched U.S. Department of Energy (DOE) *Chlorella* sp. recipient strain 1412 was shown to tolerate temperatures from 4.4 - 43.3°C (40 - 110˚ F) (Sayre et al., 2015). Different *Chlorella* species prefer different growth temperatures, with *C. vulgaris* preferring 28 - 32°C while *C. sorokiniana* prefers higher temperatures of 36 - 42°C. When *Chlorella sp.* strain DOE1412 was used as a model strain by the National Alliance for Advanced Biofuels and Bioproducts (NAABB), it was found that *C. sorokiniana* exhibits much higher maximum specific growth rates at optimal temperatures and greater thermal tolerance than other species (Sayre et al., 2015).

Some *Chlorella* species are highly salt tolerant. For example, *C. autotrophica* can survive growing in external salinities of 1 - 400% artificial seawater although cell division ceases above the 400% salinity level. This species even survived the complete evaporation of seawater. Seawater has a salinity of about 35 g/L (3.5%) of dissolved salts which are predominately sodium ions (Na^+) and chloride ions (Cl^-). Another example of a salt tolerant *Chlorella* is one investigated by Nurachman et al. (2015), *Chlorella* PP1, that was maintained in the laboratory in an artificial seawater medium.

2. The species *Chlorella sorokiniana*

As summarized in the Taxonomic Identification Report (Peñalva-Arana, 2018) *C. sorokiniana* can be distinguished from other Trebouxiophyceae using the ITS2 gene sequence (Neofotis, 2016), and by comparison of the chloroplast genomic DNA (Lemeiux, 2014). Of note and with respect to Figure 1 of the TERA application (R-18-0001), Rosenberg et al. (2014) used strains of *C. variabilis* that cluster within the group that some suggest are the true Chlorella. The tree provided shows that *C. sorokiniana* clusters separately from *C. vulgaris* and *C. variabilis* strains, its closest neighbors in that study.

As discussed in an EPA report on *Chlorella sorokiniana* developed to assist in this TERA review (Nguyen, 2017) the species *Chlorella sorokiniana* is a unicellular freshwater green alga with a characteristic emerald-green color and a pleasant “grass odor” due to high chlorophyll content. It was first found and described by the Dutch microbiologist Martinus Beijerinck in 1890. Later, it’s physiology and growth were investigated by Constantine Sorokin and Robert Krauss in 1959 and 1962. At the time, it was known as *Chlorella pyrenoidosa*.

C. sorokiniana are small cells described as spherical/ellipsoidal, about 2-6 µm in diameter, with shallow, bowl-shaped chromatophores, with the presence of pyrenoids (Shihira and Krauss, 1965; Krienitz et al., 2004). *C. sorokiniana* is a unicellular, green alga that has been used as a model organism for photosynthesis studies and in various practical applications in agriculture, biotechnology, and the food industry. Strains of *C. sorokiniana* are generally observed to be non-flagellate cells but contain a vestigial flagellar apparatus. Reproduction is often described as being achieved by producing non-motile asexual autospores (Shihira and Krauss, 1965; Luo et al., 2010). Sexual reproduction has not been reported in the literature to date but the submitters states in a previous TERA (R-17-0002) that it can be induced in the laboratory.

The following (Figure 1) is an image of *C. sorokiniana* taken from TERA submission R-18-0001.

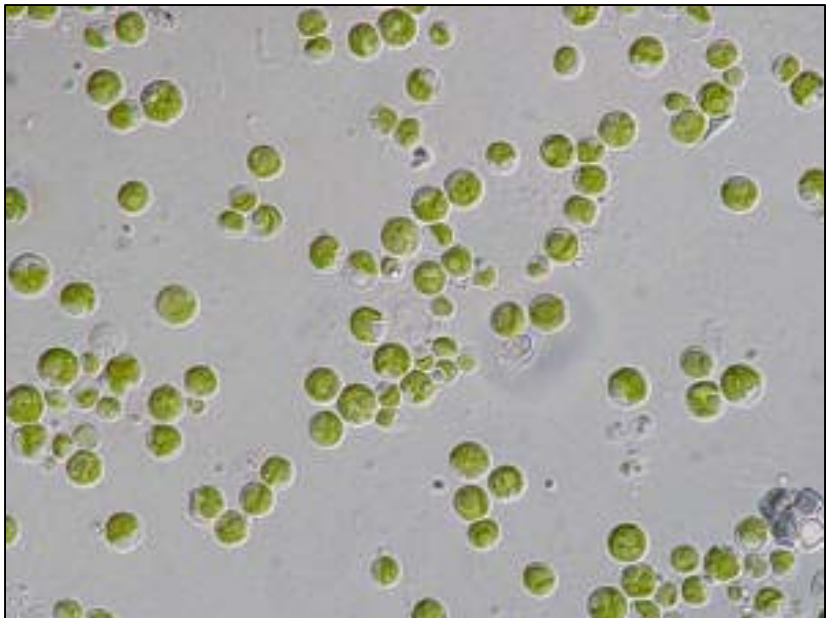

Figure 1. *Chlorella sorokiniana* cells (TERA R-18-0001)

Most *Chlorella* have a polysaccharide cell wall containing a sporopollenin-like substance that occurs in the walls of the pollen grains of higher plants. Sporopollenin is a polymer that comprises the tough outer (exine) walls of spores and pollen grains. This compound is chemically very stable and is resistant to acid and alkaline attack, enzymatic hydrolysis, and acetolysis (Ueno, 2009).

Depending on the strain, very rapid growth has been reported, with a doubling time of anywhere from 4-6 hours in phototrophic conditions (Janssen et al., 1999) and even faster in mixotrophic conditions (Wan et al., 2012).

Some strains of *C. sorokiniana* are heat tolerant. Notably the first isolated strain of *C. sorokiniana* was originally identified as a high temperature variant of *C. pyrenoidosa* (Sorokin and Myers, 1953). Several papers have shown that *C. sorokiniana* can grow at relatively high temperatures of 35 -40°C (Morita et al., 2000a, 2000b; Yamamoto et al., 2003). According to de-Bashan et al. (2008), *C. sorokiniana* is the most heat and light resistant species in the genus *Chlorella.* The strain studied, *C. sorokiniana* strain UTEX 2805, can even grow well at temperatures above 40˚C after a short adaptation period (de-Bashan et al., 2008).

Naturally occurring strains of salinity tolerant *C. sorokiniana* also exist. Mansfeldt et al. (2016) reported that an unclassified marine strain of *Chlorella* sp.*,* C596, tolerated a wide range of salinities up to that of seawater. This strain, shown to be most closely related to *C. sorokiniana,* grew better in seawater than in diluted salt concentrations. A strain of *C. sorokiniana* (HS1) originally isolated from swine wastewater with relatively high salinity levels was able to grow in all of the salinity ranges tested, up to a sodium chloride concentration much greater than that of seawater, 60 g/L (Kim et al., 2016). Chen et al. (2013) showed that a strain of *C. sorokiniana* (CY1) isolated from freshwater grew better in 10 and 20% deep seawater and could even survive in 100% deep seawater, albeit with reduced growth. Deep seawater refers to seawater at a depth of at least 200 meters that circles the globe and up-wells regularly in oceans and seas.

As discussed in the Ecological Hazard Assessment (Nguyen, 2018b), *C. sorokiniana* has been investigated as a health food due to its high carotenoid content and various vitamins (Cordero et al., 2011). In addition, due to its high lipid content compared to other algae (see Table 1), and its ability to grow under a variety of conditions, *C. sorokiniana* has been studied extensively for production of biofuels and for wastewater remediation. *Chlorella* is not only a good genus for basic research, but it is also considered as a superfood in part due to its protein content. It is also likely an important alga in the development of third generation biofuels for its lipid content, and for medical treatments (Kumar et al., 2015; Pienkos and Darzins, 2009). Table 1 gives the percentage of various components of some algae that have been investigated for potential uses including several *Chlorella* species.

Table 1. Chemical Composition of Algae - (% on dry matter basis).
(from http://www.oilgae.com/algae/comp/comp.html – source Becker, 1994)

Strain	Protein	Carbohydrate	Lipid	Nucleic acid
Scenedesmus obliquus	50 - 56	10 -17	12 -14	3 - 6
Scenedesmus quadricauda	47	-	1.9	-
Scenedesmus dimorphus	8 -18	21 - 52	16 - 40	-
Chlamydomonas reinhardtii	48	17	21	-
Chlorella vulgaris	**51 - 58**	**12 - 17**	**14 - 22**	**4 - 5**
Chlorella pyrenoidosa	**57**	**26**	**2**	**-**
Spirogyra sp.	6 - 20	33 - 64	11 - 21	-
Dunaliella bioculata	49	4	8	-
Dunaliella salina	57	32	6	-
Euglena gracilis	39 - 61	14 - 18	14 - 20	-
Prymnesium parvum	28 - 45	25 - 33	22 - 38	1 - 2
Tetraselmis maculata	52	15	3	-
Porphyridium cruentum	28 - 39	40 - 57	9 -14	-
Spirulina platensis	46 - 63	8 - 14	4 - 9	2 - 5
Spirulina maxima	60 - 71	13 - 16	6 - 7	3 - 4.5
Synechococcus sp.	63	15	11	5
Anabaena cylindrica	43 - 56	25 - 30	4 - 7	-

Chlorella has been identified as a candidate genus for development as a biofuel feedstock by the Department of Energy (DOE) (Sayre et al., 2015), as algal lipids are considered an ideal feedstock for transportation fuels (Pienkos and Darzins, 2009). Therefore, the NAABB consortium has specifically sequenced the genome of *C. sorokiniana* to facilitate the rapid development of nuclear transformation systems for this alga. Also, basic research on lipid production and biomass productivity has been conducted for various *Chlorella* strains.

Microalgae, depending on specific species characteristics and culture conditions, will employ different metabolic pathways for growth. Kim et al. (2013) reported that *Chlorella sorokiniana* may be capable of growth under autotrophic, heterotrophic and mixotrophic conditions (see Table 2). Under autotrophic

conditions, microalgae fix CO_2 to organic matter using light energy which results in the reduction of CO_2. Heterotrophic microalgae can grow using organic carbon as a sole carbon source without the need for light. Mixotrophic microalgae can metabolize both organic and inorganic carbon using metabolic characteristics of both auto- and heterotrophs. Requirements for nitrogen and phosphorus seem to also differ between all three growth types. For example, Kim et al. (2013) reported higher nitrogen and phosphorus requirements under heterotrophic growth conditions than for auto- or mixotrophic growth conditions.

Table 2. Energy and carbon source of microalgae by growth type (adapted from Kim et al., 2013).

Growth type	Energy Source	Carbon Source
Autotroph	Light	Inorganic
Heterotroph	Organic	Organic
Mixotroph	Light and organic	Inorganic and organic

Autotrophic microalgae growth has been shown to be lower than that of heterotrophic or mixotrophic types; thus, making it possible and advantageous to grow microalgae at high rates in lightless conditions. Kim et al. (2013) demonstrated this to be true for *C. sorokiniana* with hetero- and mixotrophic conditions resulting in a two times higher growth rate. The most important factor for the growth of any autotrophic culture is light intensity, while organic carbon as a sole carbon source significantly affects the growth of heterotrophic microalgae. Therefore, Kim et al. (2013) suspected that the slower growth rate under autotrophic conditions was due to photoinhibition due to high cell density. This has been shown to be the case for *C. sorokiniana* when comparing heterotrophic versus autotrophic growth at high densities (Zheng et al., 2012).

C. sorokiniana's overall essential nutrient requirements do not differ greatly from that of other microalgae. Eyster (1967) reported on the most suitable growth conditions for *C. sorokiniana* when grown in a photobioreactor (PBR), and went on to develop an optimal medium for their growth at temperatures of 25 and 39˚C. He reported that minimum nutrient concentrations varied between 2-5 times higher at the higher temperature, except for phosphorus which required a concentration 25x higher, and it was hypothesized this might be related to the thermotolerant nature of *C. sorokiniana.* Eyster (1967) also reported that *C. sorokiniana* cultures exhibit maximum growth in the pH range of 6.15 - 6.3. He also reported that when grown at higher densities, *C. sorokiniana* is more tolerant of shifts in pH. Nutrients required for the growth of *C. sorokiniana* included nitrogen, phosphorus, magnesium, sulfur, potassium, iron, calcium, manganese, zinc, and copper. However, his study gave no indication that *C. sorokiniana* requires boron, sodium, cobalt, molybdenum or chloride for growth.

B. Donor Microorganisms

The intergeneric gene used to develop the strains in this TERA, the *SNRK2* gene was derived from a *Picochlorum soloecismus* strain, a genus of green algae in the class Trebouxiophyceae. *Picochlorum soloecismus* is a halotolerant, fast-growing and moderate lipid producing microalga that has been evaluated as a renewable feedstock for biofuel production by the DOE (Gonzalez-Esquer et al., 2018).

The sucrose non-fermenting related kinase 2 (SNRK2) is part of the serine/threonine kinases (Kertesz et al., 2002) and plays a key role in sugar metabolism in plant and animal kingdoms and controls multiple growth and metabolic processes. Members of the SNRK2 subclasses have been studied primarily in plants (e.g., *Arabidopsis*) and are implicated in both direct and indirect abscisic acid (ABA) response

pathways dealing with environmental stress-signaling (Holappa et al., 2017; Todaka et al., 2015). Although the ABA signaling pathways have been extensively studied in plants, knowledge of their roles in algae and other lower photosynthetic species (e.g., cyanobacteria and lichens) remain limited. ABA synthesis in algae however is known to be induced by environmental stressors like drought or salt stress (Hartung, 2010). It has been shown that all SNRK2 subclasses are well conserved among higher plants, yet SNRK2s in algae (e.g., *Chlamydomonas*) have been classified as having distinct sequences from those found in higher plants (Hauser et al., 2011).

Per the submitter, the SNRK2 enzyme plays a critical role in *Arabidopsis* plants to regulate the energy metabolism. When overexpressed in *Arabidopsis*, *SNRK2* conferred increased sucrose synthesis, starch synthesis, and leaf growth (Zheng et al., 2010). The SNRKs have also been detected in almost all streptophyte algae (de Vries et al., 2018), and implicated with cold stress adaptation for the alga *Chlamydomonas reinhardtii* (Valledor et al., 2013). Streptophyte algae are a small group of freshwater algae ranging from scaly, unicellular flagellates (*Mesostigma*) to complex, filamentous thalli with branching, cell differentiation and apical growth (Charales). Streptophyte algae and embryophytes form the division Streptophyta, whereas the remaining green algae are classified as Chlorophyta (Becker and Marin, 2009).

The *SNRK2* gene from *P. soloecismus* was synthesized in its native state (only the coding regions) without codon optimization and cloned into the PACE_Chlorella_Zeocin_Plasmid vector. This plasmid was specifically developed for use in genetically modifying green algae/*Chlorella*, and have been used for many years by PACE, including in a previous TERA submission (formerly listed as PACE_Chlorella_Plasmid, R-17-0002). The submitter expected the overexpression of *SNRK2* would improve starch accumulation and growth in *Chlorella* cells. Compared to wild-type *C. sorokiniana* 1412, the submission microorganism PACE_Cs1412_SNRK2 showed an increase in biomass, starch accumulation, and photosynthetic efficiency.

The chemically synthesized, codon-optimized Sh *ble* gene (previously accessed for a TERA submission R-17-0002, that confers resistance to the antibiotic zeocin is based on the sequence found in the bacterium *Streptoalloteichus hindustanus* ATCC 31158. This marker gene enables selection of transformants from culture medium containing zeocin (Gatignol et al., 1988; Drocourt et al., 1990). *Streptoalloteichus* is a genus of bacteria within the family *Pseudonocardiaceae*. This organism produces antitumor antibiotics known as tallysomycins which belongs to the bleomycin (BLM) family of antitumor antibiotics (Tao et al., 2007).

III. ECOLOGICAL INTERACTIONS OF ALGAE IN THE ENVIRONMENT

The interactions of algae in aquatic and terrestrial environments and their role in aquatic food webs were discussed in a previous risk assessment for an algal submission by McClung (2013).

A. Aquatic Ecosystems

A number of factors affect the rise and fall of algal populations in the aquatic environment including the physical factors of light, temperature, weather, water movements, flotation, the chemical nutrient status of nitrogen, phosphorus, silicon, calcium, magnesium, potassium, sulfate, chloride, iron, manganese, and other trace elements, and organic matter (Ikawa, 2004). There are a number of

biological factors as well including the presence of resting stages, predation, and parasitism. The polyunsaturated fatty acids produced by algae can affect algal growth. In addition, a number of biological substances are known to be produced by algae that inhibit the growth of other algal or of zooplankton grazers (Pratt et al., 1944; Pratt et al., 1945). Likewise, it has been shown that some algae detect "infochemical" signals from grazers, and can change their morphology accordingly to try to avert predation (Lass and Spaak, 2003).

Algae and cyanobacteria are the basis of the food web in both freshwater and marine aquatic ecosystems. Food webs in water bodies are complex and dynamic and have been shown to vary from season to season and with other perturbations of the water body, e.g., eutrophication (Lindeman, 1942; Martinez, 1991). The phytoplankton community of a typical north-temperate lake has been shown to consist of up to several hundred algal species that co-exist (Kalff and Knoechel, 1978). Phytoplankton diversity is influenced not only by the different ecological niches within a water body (e.g., benthic vs. pelagic regions), but also by a number of temporal and spatial variations in factors such as nutrient supply, temperature, dissolved oxygen, predation, and parasitism (Wehr and Sheath, 2003; Townsend et al., 1998). Nutrient supply and herbivory are thought to be the most important parameters affecting diversity changes over time.

According to Wehr and Sheath (2003), the phytoplankton species composition in lake food web ecosystems is important because the 'functional properties of algal assemblages vary strongly with species composition'. Different taxa are important because features that are sometimes used to classify various species such as photosynthetic pigments, storage products, motility, reproduction, cell ultrastructure, and even DNA sequence have functional importance in the ecosystem. For example, nitrogen fixation by phytoplankton is of great functional importance but is restricted to a limited number of cyanobacteria. Also, photosynthetic pigment production is important. For instance, the presence of the red accessory pigment phycoerythrin with an absorption maximum of 540-560 nm broadens the photosynthetic capacity of an ecosystem by facilitating growth at greater depths (Goodwin, 1974). Autotrophic picoplankton have a strong competitive advantage under phosphorus-limiting conditions (Suttle et al., 1998; Wehr, 1989).

Diversity in the size fractions of phytoplankton is an important aspect of algal communities and thus food webs (Bott et al., 1996). For planktonic food webs, cyanobacteria have a dominant role in aquatic productivity. It is these smaller autotrophs that provide excreted dissolved organic compounds that provide substrates for heterotrophic bacterial growth. In addition, cyanobacteria are directly grazed by protozoa (microflagellates and ciliates). This microbially-based food web in which the major portion of autotrophic production occurs is important to the marine food webs. The microbial food web consists of those organisms that are < 1000 µm, and in freshwater benthic ecosystems consists of (presented by increasing size fraction) cyanobacteria and bacteria, followed by microflagellates, diatoms and green algae, which are then consumed by ciliates, rotifers, copepods, oligochaetes, nematodes, and then invertebrate macrofauna followed by the larger vertebrates (Bott, 1996). A complex microbial food web has bacteria and algae at the lowest trophic level, which are then consumed by protozoa and meiofauna which are organisms in the size range of approximately 50 - 1000 µm including large ciliates and metazoa (e.g., rotifers, copepods, and oligochaetes). An important link between microbial food webs and classical food webs are the autotrophic picoplankton (> 0.2 - 2 µm). These cyanobacteria are grazed mainly by micro-zooplankton (ciliates, flagellates) rather than by cladocerans or copepods (Pernthaler et al., 1996; Hadas et al., 1998). Size also affects the sinking rate with smaller planktonic species sinking more slowly. Thus, the smaller species remain more prevalent in the euphotic zone.

B. Terrestrial Ecosystems

Algae occur in nearly all terrestrial environments on earth, including desert soil crusts, and are invariably encountered on and beneath soil surfaces (Metting, 1981). Acceptance of algae as bona fide soil microorganisms occurred late in the 19th century when it was recognized that certain groups were restricted to soil, including some *Chlorella* species (Shihira & Krauss, 1965; Kessler, 1976). Over 38 prokaryotic genera and 147 eukaryotic genera have been identified as terrestrial species, the majority of which are truly edaphic. As expected, solar radiation, water, and temperature are the most important abiotic factors controlling their distribution, metabolism, and life histories (Metting, 1981). Biotic interactions are also important, but much less well understood. Algae play an important role in primary and secondary plant community succession by acting as an integral part of ecosystem. Algal communities living in soil have the principal function of primary productivity, nitrogen fixation, and stabilization of aggregates, i.e., prevention of soil erosion (Metting, 1981). Algae concentrations in soils are typically found to be between 10^3 and 10^4 cells/gram dry soil but have been reported as high as 10^8 (Metting, 1981).

IV. DISPERSAL OF ALGAE IN THE ENVIRONMENT

Algae can be dispersed in the environment by a number of different mechanisms including water, wind, and by aquatic and terrestrial organisms. These mechanisms and organisms of dispersal were discussed in a previous algal risk assessment by McClung (2013).

As previously mentioned, *Chlorella* are ubiquitous in the environment in freshwater, marine environments, and in soils. The occurrence of many species of algae throughout the world suggests that algae can readily disperse over great distances. Studies on microalgae have shown that most species are globally distributed (cosmopolitan) but some species have more restricted distribution due to environmental factors such as temperature or humidity, and limited dispersal mechanisms (Kristiansen, 1996a). In a review of data on the distribution of coccoid green algae in the environment, Komárek and Comas (1984) reported that the distribution is dependent on the specific environmental requirements of the taxon. They stated that "Chlorococcalean algae (*Chlorella* belongs to this group) are traditionally supposed to be organisms of cosmopolitan occurrence. Many species occur, indeed, in various regions all over the world, but, many other taxa occur in geographically limited areas, mainly in either the northern or the tropical countries". *C. sorokiniana* has been collected from various biome types across Africa. For example, *C. sorokiniana* has been found in biological soil crusts across Africa's Kalahari's dry savanna, in Namibia's succulent Karoo, and in Zambesian's dry forest (Budel et al., 2009). This demonstrates *C. sorokiniana's* ability to survive in highly diverse ecosystems with extreme temperature ranges. One strain of *C. sorokiniana* was reported to have an optimal growth at temperatures between 38-42˚C (Kessler, 1985) while others report that it can be grow at temperatures ranging from 14-38°C (Patterson, 1970). *C. sorokiniana* is capable of surviving temperatures encountered in hot deserts.

A. Dispersal by Water

Passive dispersal of algae by water can occur wherever there is running water between connected water bodies. A study by Atkinson (1988) found that the colonization of a newly constructed reservoir was

from the inflow, and it took several years before there was the appearance of organisms different from those found in the catchment area. Heavy precipitation and flooding can result in algal dispersal by connecting water bodies that are usually isolated. Algal dispersal by water is likely more important in wetter environments that in arid regions.

B. Dispersal by Aerosols

Air currents are an important dispersal mechanism for algae, and it is thought that algae have spread throughout the globe as aerosols. As early as 1844 Ehrenberg recognized the presence of airborne algae in dust samples collected 300 km off the nearest coast by Darwin in 1939 on the H.M.S. Beagle (Kristiansen, 1996b).

According to a review article by Sharma et al. (2007), "In general, bioaerosols range from 0.02 to 100 µm in diameter and follow the same physical rule as any particle of a similar aerodynamic diameter. They disperse via air movements and settle according to the settling velocity, available impaction, surface, and climatic factors prevailing in the area (Burge and Rogers, 2000)". Air movements within a laminar boundary layer surrounding the source usually release such particles. Many of the particles remain in the layer and eventually settle near the source (<100 m), while some are carried aloft with turbulence and transported by the wind over a long distance. The processes responsible for the release and atomization of bioaerosols from natural sources are as follows:

1. Sweeping of the surface or rubbing together of adjacent surfaces by wind and gusts dislodges the bioparticles from the surface. Dried algae caught by the wind are carried away like dust particles (Grönblad, 1933; Folger, 1970).

2. Formation of oceanographic aerosols by wave action and the bursting of bubbles at the water-air interface (Woodcock, 1948; Stevenson and Collier, 1962; Maynard, 1968; Schlichting, 1974). Fragments of scums and foams with algal contents along the shoreline of water bodies can be picked up by the wind and carried aloft (Maynard, 1968).

3. During heavy rainfall, algae are splashed up by raindrops and can be entrained into the atmospheric air by thermal winds (Burge and Rogers, 2000).

4. Storm activity over land and sea where great turbulence is experienced.

5. Human activities, such as agricultural practices, construction and maintenance practices, sewage treatment plants (Mahoney, 1968, as cited in Sharma et al., 2007), garbage dumping, highway traffic, and to a limited extent weapons testing and spacecraft launching, can result in the atomization of constituting algae (Schlichting, 1974; Kring, 2000).

6. Atomization of aerosols to a low height also occurs when surface water containing blooms is used for irrigation and recreational activities like boating, jet skiing, and so forth (Benson et al., 2005)".

Sharma et al. (2007) also stated, based on the result of earlier publications, that green algae, cyanobacteria, diatoms, and tribophytes comprised most of the aero-algae flora. Cyanobacteria dominate the aero-algae flora of tropical regions whereas chlorophytes (green algae) dominate in the temperate regions.

Brown (1964) conducted studies on airborne algae using agar petri dishes suspended in stationary locations in Texas, and impaction studies of algae onto agar petri dishes collected from moving automobiles in 14 states. He also collected samples from an airplane. The impaction from the moving automobiles and planes yielded the greater numbers and diversity of algae. For example, the agar plates held from a moving car in Pennsylvania yielded 140 algal impactions composed of approximately 25 different genera of algae. A 10-second exposure obtained from a moving car sampling a local dust cloud resulting from plowing of a field recorded 5000 algal compactions, of which 4500 were chlorophycean or xanthophycean. *Chlorella* was one of the algal genera found. The algal content of dust was found to be quite high at > 3000 cells per m^3. The author concluded that soil is the predominant source of airborne algae.

Schlichting (1969) conducted studies on airborne algae in Michigan and Texas using Millipore filters and bubblers containing soil-water extracts at heights of 6, 15, 30, 75, and 150 feet from the ground. Also, aerial sampling of maritime algae was made from a ship 100 miles off the coast of North Carolina. Over an eight-year period, the number of algae collected never exceeded 8 cells/ft^3. He then estimated that a person at rest would inhale 240 algal cells per hour which would result in an inhalation exposure of approximately 2880 cells/day. Higher algae numbers were found in the Texas samples from dust than those from water environments. In a summary of the existing literature on algae found in aerosols, viable cells of *Chlorella* were sampled directly from the air in these states and in Holland and Taiwan. Species of *Chlorella* found included *C. ellipsoidea, C. pyrenoidosa, C. vulgaris*, and *Chlorella* sp. (Schlichting, 1969).

The diversity and abundance of airborne green algae and cyanobacteria on monuments and stone art works in the Mediterranean Basin was studied by Macedo et al. (2009). Airborne *Chlorella, Stichococcus,* and *Chlorococcum* were the three most frequently encountered chlorophyte.

The diversity of aero-algae in a Mediterranean river-reservoir system was found to be high (Chrisostomou et al., 2009). They found that nanoplanktonic algae comprised the majority (46.4%) of the aero-algae flora with *Chlorella* being the predominant aero-alga. Three of the most frequently isolated nanoplanktonic airborne algae were *Chlorella vulgaris, Didymocystis bicellularis,* and *Scenedesmus obliquus.* The authors suggested that these vegetative cells have a protective external coating that allows them to resist desiccation in bioaerosols for short distances.

The cell wall of algae is thought to contribute to its ability to survive in aerosols. The cell walls of freshwater and marine algae are composed of a highly resistant biopolymer called algaenan which is a large component of kerogen, the organic matter of sedimentary rock (Blokker et al., 1998). Type I kerogen is largely composed of algal cell wall remains due to the highly resistant nature of algaenan. In addition, as previously mentioned, the cell walls of *Chlorella* also contain sporopollenin (Atkinson et al., 1972). Sporopollenin is a compound that is chemically very stable and resistant to acid and alkaline attack, enzymatic hydrolysis, and acetolysis (Ueno, 2009).

Genitsaris et al. (2011) did a comprehensive review of studies in the published literature on airborne algae. They summarized that the most frequently occurring algae isolated from aerosols were *Chlorella, Scenedesmus, Chlorococcum,* and *Klebsormidium,* and the cyanobacterium *Lyngbya.* These species were found in more than 40% of the sites that had been sampled by various researchers in their aero-algae studies. Soil bound *Chlorella* species appear to tolerate higher levels of radiation than other more complex terrestrial life forms (Metting, 1981). Trainor (1962) was able to show that *Chlorella* is able to

survive desiccation for one hour at 130°C.

As noted in the Ecological Hazard Analysis for a previous TERA (R-17-0002) (Peñalva-Arana, 2017), recent work by Szyjka et al. (2017) demonstrated that cultivation of the genetically engineered (GE) alga *Acutodesmus dimorphorus* (formerly *Scenedesmus dimorphous*) in outdoor miniponds resulted in transport of the algal cells in bioaerosols. Their data showed that the algal cells transport was a function of distance and wind direction. Using qPCR to detect both the wildtype and GE strains, detectable levels of both were found in trap buckets at distances from 5-50 meters away. However, neither strain was able to outcompete local or airborne algae taxa in either the trap buckets or in laboratory experiments conducted using local eutrophic and oligotrophic lake water containing local taxa. Their research also showed that airborne algae have high diversity (species detected using ITS2 primers) and many species, including their test species, could establish in waters with naturally occurring algal communities.

In aquatic habitats, microorganisms are known to be concentrated in the surface films and in foams on the water surfaces (Maynard, 1968). Schlichting (1974) conducted studies on the ejection of microorganisms into the air with bursting bubbles. He found that bubbling air through a bacterial culture resulted in 2,000 times more bacteria in the bubble jet droplets. Microorganisms in the range of 0.3 to 30 µm in diameter can be carried in atmospheric water droplets (Woodcock, 1948, as cited by Schlichting, 1974).

Airborne algae are subject to desiccation stress and ultraviolet light exposure (Sharma et al., 2007). Desiccation, the equilibration of an organism to the relative humidity of the surrounding atmosphere, is an intensive stress that typically, most phototrophic organisms cannot survive (Holzinger and Karsten, 2013). However, there are studies that suggest that some algae can survive desiccation stress (Evans, 1958, 1959; Schlichting, 1961). A comprehensive list of algae capable of surviving desiccation was published in 1972 by Davis. Parker et al. (1969) reported that various cyanobacteria and green algae survived desiccation as viable algae were found in decades-old air-dried soil samples. This is in contrast to Schlichting (1960) who reported survival of only four hours with desiccation stress. Ehresmann and Hatch (1975) studied the effect of relative humidity (RH) on the survival of the unicellular eukaryotic alga *Nannochloropsis atomus* and the prokaryotic alga *Synechococcus* sp. Viable cells of the latter species could be recovered at all the RHs tested (19 - 100%). However, there was a progressive decrease in the number of viable *Synechococcus* cells with lower RHs. There was a stable survival at RH 92% and above. The results with the eukaryotic green alga were very different. No viable cells of *N. atomus* were recovered below 92% relative humidity.

In an earlier study Schlichting (1961) found that algae remained viable under a wide range of environmental conditions including RHs of 28-98%. The stress associated with atomization of the algae was responsible for rapid decrease in viability. So perhaps, the gradual air-drying of soil samples as in Parker et al. (1969) did not result in death of the microorganisms. Slow desiccation to an air-dried state was shown to facilitate faster growth recovery of *Scenedesmus dimorphus* and the marine alga *Nannochloropsis* than fast desiccation, although both strains showed significant growth inhibition after desiccation (Anadarajah et al., 2011).

C. Dispersal by Aquatic and Terrestrial Organisms

Aquatic and terrestrial organisms are also responsible for algal dispersal (Kristiansen, 1996b). Even fish can act as vectors. For example, numerous species of plankton algae including cyanobacteria, green

algae, and diatoms have been found to pass undamaged through the digestive track of the plankton-eating gizzard shad (Velasques, 1940 as cited by Kristiansen, 1996b). Insects such as beetles have been found to carry viable algae in their digestive tract (Parsons et al., 1966, as cited by Kristiansen, 1996b), and thus, their fecal pellets can distribute algae to new water bodies. Milliger and Schlichting (1968) found 20 species of green algae in the intestinal tract of beetles. Algae dispersal by beetles is a likely mechanism for small water bodies for short distances (Kristiansen, 1996b). Other insects can disperse algae to various water bodies. Revill et al. (1967) found that with four species of aquatic Diptera (craneflies and midges), 21 different genera of algae were found on the collected insects. Likewise, Sides (1968) found that the mud dauber wasp was capable of carrying algae and protozoa as nine and four genera, respectively, were isolated from aseptically collected insects. Parsons et al. (1996, as cited by Kristiansen, 1996b) reported the presence of 20 genera of viable blue-green algae (currently cyanobacteria), green algae, and euglenoids in and on dragonflies and damselflies. Dragonflies are thought to be able to transport algae possibly long distances (Maguire, 1963).

Water-living mammals and other mammals such as mink, muskrats, and raccoons can transport viable algae on their fur and sometimes in their intestinal tracts. Human activities (e.g., boating, fishing, hunting) can also transport algae between water bodies. For instance, the use of felt-soled wading boots has been banned in a number of states as they have been shown to transport non-native larvae, spores, and algae between water bodies. In Vermont, the felt-soled wading boots are believed to have spread didymo, a slimy alga also called rock snot, to various rivers throughout the state. This alga forms dense mats that blanket the bottom of the stream like a shag carpet, changing pristine trout streams to a green, yucky mess, according to a fisheries biologist with the state Fish and Wildlife Department (http://usatoday30.usatoday.com/news/nation/environment/2011-04-28-rock-snot-felt-sole-wader-ban_n.htm).

D. Dispersal by Birds

Water birds are one of the most important vectors for algae dispersal as they can transport live algae on their feet and feathers and sometimes internally in their bills or in their digestive tract (Kristiansen, 1996b). Water birds such as seagulls have been shown to transport algae, particularly aquatic desmids, in wet mud on their feet for long distances (Strøm, 1926). Desiccation is of course of great importance with the viability of live algae transported on the feathers or feet of birds. Algae carried internally in the digestive tract are not subject to desiccation stress.

Migratory birds have a significant role in the transport of algae for long distances (Kristiansen, 1996b). Proctor (1959) studied the carriage of algae in the intestinal tract of numerous migratory bird species obtained from playa lakes in Texas and Oklahoma. A number of freshwater algae species were found in the alimentary canal of 25 different migratory birds. Algae were found in the lower digestive tract of the pied-bill grebe, the green-winged teal, the blue-winged teal, the shoveler, the American coot, the killdeer, the dowitcher, the American avocet, the Wilson's phalarope, and the belted kingfisher. Since many species of blue-green algae (currently cyanobacteria) and green algae do not have spores or specialized resting structures, the algae were assumed to have been transported as vegetative cells. Based upon the rate of movement of the algae through the alimentary tract and the flying speed of some common migratory birds, Proctor (1959) suggested that algae could be easily transferred between lakes 100 - 150 miles apart, with much greater distances possible with cells or colonies in the caecum of the birds.

Schlichting (1960) also investigated the transport of algae on and in various waterfowl. He measured the carriage of chlorophyta (green algae), cyanophyta (blue-green algae), chrysophyta (golden algae), euglenophyta, bacteria, fungi, protozoa, and rotifers and on the feet and feathers, and in the bill and gullet, as well as in the fecal matter of 105 birds representing the following 16 species of waterfowl: black duck (*Anas rubripes*), blue goose (*Chen caerulescens*), buffle-head duck (*Bucephala albeola*), Canada goose (*Branta canadensis*), coot (*Fulica americana*), Eastern belted kingfisher (*Megoceryle alcyon*), gadwall *(Anas strepera*), goldeneye (*Glaucinetta clangula americana*), green-winged teal (*Anas carolinensis*), mallard (*Anas platyrhynchos*), redhead duck (*Aythya americana*), ring billed gull (*Larus delawarensis*), ruddy duck (*Oxvura jamaicensis*), spotted sandpiper (*Actitis macularia*), common snipe (*Capella galinago*), and wood duck (*Aix sponsa*).

The field collection experiments demonstrated that the water birds retained viable forms of algae and protozoa both externally and internally. For those organisms carried externally on the feet and feathers, the birds exposed to the air for less than four hours carried a great variety of organisms. Those exposed to air for longer periods of time had fewer viable organisms. With eight hours of exposure to air, there were some organisms on the feet of birds, but a greater variety was found to be carried in the bills. The birds exposed to the air longer than eight hours yielded very few organisms. The contents from the gullets sampled produced good algal growth in culture, whereas only a few of the 163 fecal samples contained viable algae or other organisms. Viable organisms found on the waterfowl consisted of 86 species from the feet, 25 species from the feathers, 25 species from the bills, 14 species from the gullets, and 12 organisms from the fecal material.

The following species of green algae were found on the feet of the waterfowl: *Ankistrodesmus braunii, A. convolutus, A. falcatus, Arachnochloris-like cells, Arthrospira gomotiana, A. jenneri, Chlamydomonas globosa, C. mucicola, C. pseudopertyi, C.* sp.*, Chlorococcum* sp.*, Chlorella ellipsoidea, C. vulgaris, Chlorella* sp.*, Closteriopsis-like cells, Dactylococcopsis acicularis, Franceia* sp.*, Glenodinium* sp.*, Gloeocystis gigas, Mougeotia* sp.*, Nannochloris bacillaris, Oedogonium* sp.*, Oocystis rorgei, Palmodictyon* sp.*, Protococcus* sp.*, Rhabdoderma irregulare, Rhizoclonium fontanum, Scenedesmus abundans, S. dimorphus. S. quadricauda, Scenedesmus* sp.*, Sphaerocystis, Schroeteri, Tetraedron minimum, T. sisconsinense, Tetraedron* sp.*,* and *Ulothrix* sp. The cyanobacteria found on the feet included the following species: *Anabaena affinis, Aphanocapsa* sp.*, Aphanothece castagnei, A. nidulans, Chroococcus dispersus, C. minutus, Gloeocapsa* sp.*, Gloeothece linearis, Lyngbya attenuata. L. limnetica, L.* sp.*, Microcystis aeruginosa, Nostoc* sp.(?), *Oscillatoria angustissima, O. limnetica, O. subbrevis, O. tenuis, O. terebriformis, Oscillatoria* sp.*, Pelo-gloea bacillifera, Phormidium mucicola, P. tenue, Phormidium* sp.*, Plectonema nostocorum,* and *Synechococcus aeruginosus.*

Much fewer numbers of green algae, cyanobacteria, golden algae, euglenoids, protozoa, and fungi were found on the feathers. Of the nine green algae species detected, there were two species of *Chlorella, Chlorella ellipsoidea* and *C. vulgaris.* Still fewer total numbers of organisms were found in the bills, but *C. vulgaris* was one of them. *Chlorella* was not any of the five green algae isolated from the gullet, nor any of the five green algae found in fecal material. It was also speculated by Schlichting (1960) that some microalgae, specifically *Chlorella*, may become embedded in the matrix of larger taxa, such as *Gloeocystis*, and be able to be transported away not only far, but also protected for greater periods of time.

V. HISTORY OF USE

The submitters previously submitted a similar TERA application in 2017 (R-17-0002), where a genetically engineered strain of *Chlorella sorokiniana* (UTEX 1230) was used. That submission was approved for the genetically engineered *C. sorokiniana* UTEX 1230 strain Cs1230-P5CS-T3 to be tested in open raceway miniponds.

However, since *Chlorella* are frequently used in dietary supplements, there is a long history of growing naturally occurring *Chlorella* strains. The genus *Chlorella* has a long history of research and experimentation. It is a ubiquitous genus that can be found in marine, freshwater and edaphic habitats making it one of the most ubiquitous and famous microalgae genus worldwide. Much of what was first discovered about the fundamentals of photosynthesis and inorganic nutrition came from experiments using *Chlorella* (Shihira & Krauss, 1965).

Various *Chlorella* species, including *C. sorokiniana*, have been extensively evaluated for their application in animal feed, human food, nutritional supplements, cosmetics, and pharmaceuticals, and more recently for biofuels (Kang et al., 2004).

VI. CURRENT USE AND FUTURE USES

The purpose of this TERA submission is to perform a time limited (60 day), defined scope R&D field experiment in outdoor miniponds. Therefore, the submitters will not be creating any formulation, product, commercial or otherwise, or have any other uses for this microorganism. The data obtained from this experiment will be used to inform future research and development activities, as well as future TERA/Microbial Commercial Activity Notices (MCAN) submissions.

Per the submitter, the *SNRK2* gene is shown to play a critical role in *Arabidopsis* plants to regulate the energy metabolism. The over-expression of *SNRK2* gene in *Arabidopsis* increased sucrose synthesis, starch synthesis and leaf growth. They expect overexpression of the *SNRK2* gene will improve starch accumulation and growth in *Chlorella* cells. For the field experiment, the submission strain has been engineered to show improved phenotypic properties of larger cell size, faster biomass accumulation and higher carbohydrate content than the parent microorganism.

According to the TERA (R-18-0001) submission, the submission microorganism will be used in this field experiment to:

1) to evaluate the translatability of GM phenotypes from a lab to an outdoor setting

2) and to compare the submission and recipient microorganisms when cultivated in outdoor miniponds with respect to their ability to produce biomass

3) and to identify any increase or decrease in biomass productivity under biotic (bacteria, predators) and abiotic (diurnal temp and light) stressors from the environment

Depending upon the results of this field experiment, it is expected that in the future this strain PACE_Cs1412_SNRK2 may be further genetically modified to produce useful products such as algal biofuels or bioproducts.

Since this is a TERA submission, the use of the submission strain PACE_Cs1412_SNRK2 is restricted solely to the conditions of use outlined in the TERA. Thus, the submitter cannot use this strain for additional field tests nor for any other purpose without submitting another TERA or an MCAN to the Agency.

VII. GENETIC MODIFICATIONS

The genetic construction and modifications of the recipient to arrive at the final strain PACE_Cs1412_SNRK2 to be field tested were analyzed by Cameron (2018).

As previously stated, the recipient microorganism is *Chlorella sorokiniana* DOE1412. The submission strain, PACE_Cs1412_SNRK2, contains an intergeneric DNA sequence that encodes the sucrose non-fermenting related kinase 2 (S*NRK2*) gene from the green alga *Picochlorum soloecismus*. PACE_Cs1412_SNRK2 also contains an intergeneric DNA sequence Sh *ble*, which is from *Streptoalloteichus hindustanus* ATCC 31158 and encodes resistance to the antibiotic zeocin (Gatignol et al., 1988; Drocourt et al., 1990).

The submission strain was produced by the following manipulations. In a series of steps, a plasmid was constructed (SNRK_PACE_Chlorella_Plasmid) and cleaved in one site via restriction digestion. The linearized plasmid was used to transform the recipient strain via electroporation. The linear DNA contained two intact intergeneric genes, *SNRK2* and Sh *ble* (a.k.a., *zeoR*). In addition, it contained the *bla* gene that encodes resistance to the antibiotic ampicillin (a.k.a., *ampR*). However, this *bla* gene was cleaved in the middle during linearization and a functional beta-lactamase is not expected to be expressed. Of note, unlike with TERA R-17-0002, the submitters did not use the backcrossing protocol in developing the submission strain of this TERA.

The *SNRK2* gene used in this TERA is a synthetic, non-codon optimized sequence based upon a gene from *Picochlorum soloecismus.* It is regulated by the synthetic versions of native *C. sorokiniana psaD* promoter and terminator. As previously mentioned, the *SNRK2* gene is shown to play a critical role in *Arabidopsis* plants to regulate the energy metabolism. The over-expression of *SNRK2* gene in *Arabidopsis* increased sucrose synthesis, starch synthesis and leaf growth (Zheng et al., 2010). The submitters expect over-expression of the *SNRK2* gene to improve starch accumulation and growth in *Chlorella* cells. SNRK2 is expressed in the cytosol and is an intracellular protein. The SNRK2 subfamily is implicated in osmotic signaling in light of the finding that all the SNRK2 kinases except SNRK2.9 can be activated by hyperosmotic and NaCl stress in *Arabidopsis* (Zheng et al., 2010). The *SNRK2* gene expressed by the submission strain has the catalytic domain of the Serine/Threonine Kinases, Sucrose nonfermenting 1-related protein kinase subfamily 2. STKs catalyze the transfer of the gamma-phosphoryl group from ATP to serine/threonine residues on protein substrates. The SNRKs form three different subfamilies designated SNRK1-3. SNRK2s are involved in plant response to abiotic stresses and abscisic acid (ABA)-dependent plant development (Marchler-Bauer et al., 2017).

The *SNRK2* gene was synthesized and cloned by a commercial firm, Genewiz (https://www.genewiz.com/en), into a PACE_Chlorella_Zeocin_Plasmid vector (shown in Figure 2). The PACE vector was previously developed by researchers at the New Mexico Consortium to introduce genes of interest into *Chlorella* sp. It was built via intermediate plasmids on the *E. coli* vector backbone from plasmid pSL18, a non-published derivative of a commercial pBS KS+ plasmid (unpublished data and personal communication between S. Cameron and S. Lemaire, the developer of the pSL18 plasmid). It contains: 1)

an *E. coli* plasmid origin of replication and an ampicillin resistance (*bla*) gene for propagation and selection in *E. coli*; 2) the *Streptoalloteichus hindustanus* Sh *ble* gene, which confers zeocin (ZeoR) resistance, under the control of the *C. sorokiniana* actin promoter/terminator; 3) an empty multiple cloning site (MCS) for insertion of the gene of interest; 4) the *psaD* promoter/terminator pair flanking the MCS; 5) a 13 bp sequence to add restriction sites to the MCS; and 6) additional sequence (several hundred nucleotides) to expand the *psaD* terminator region. This promoter was chosen due to its relatively high expression as a native photosynthesis-related gene promoter in *C. sorokiniana.*

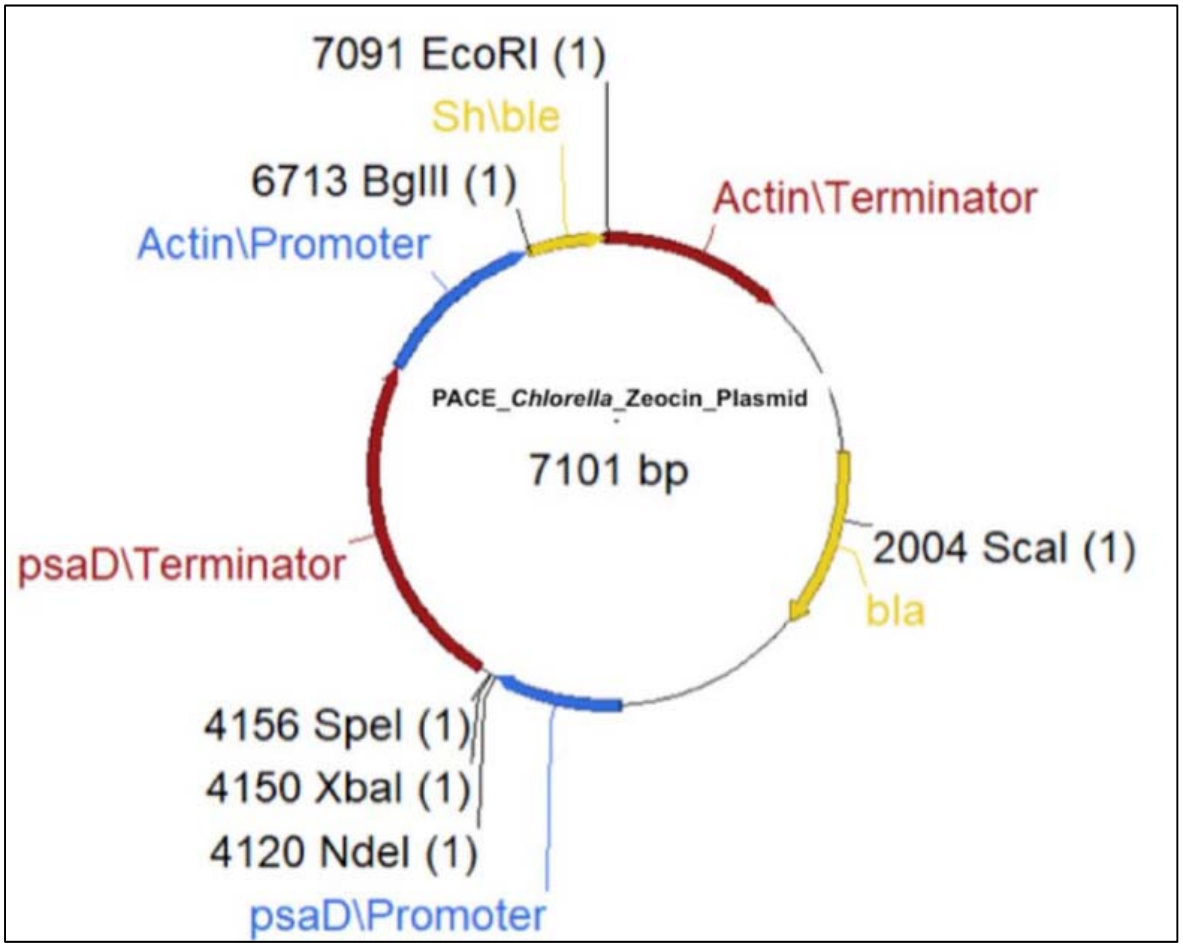

Figure 2. The PACE_*Chlorella*_Zeocin_Plasmid

The *SNRK2* gene sequence was introduced into the PACE_*Chlorella*_Zeocin_Plasmid to form the final SNRK_PACE_Chlorella_plasmid vector (Figure 3).

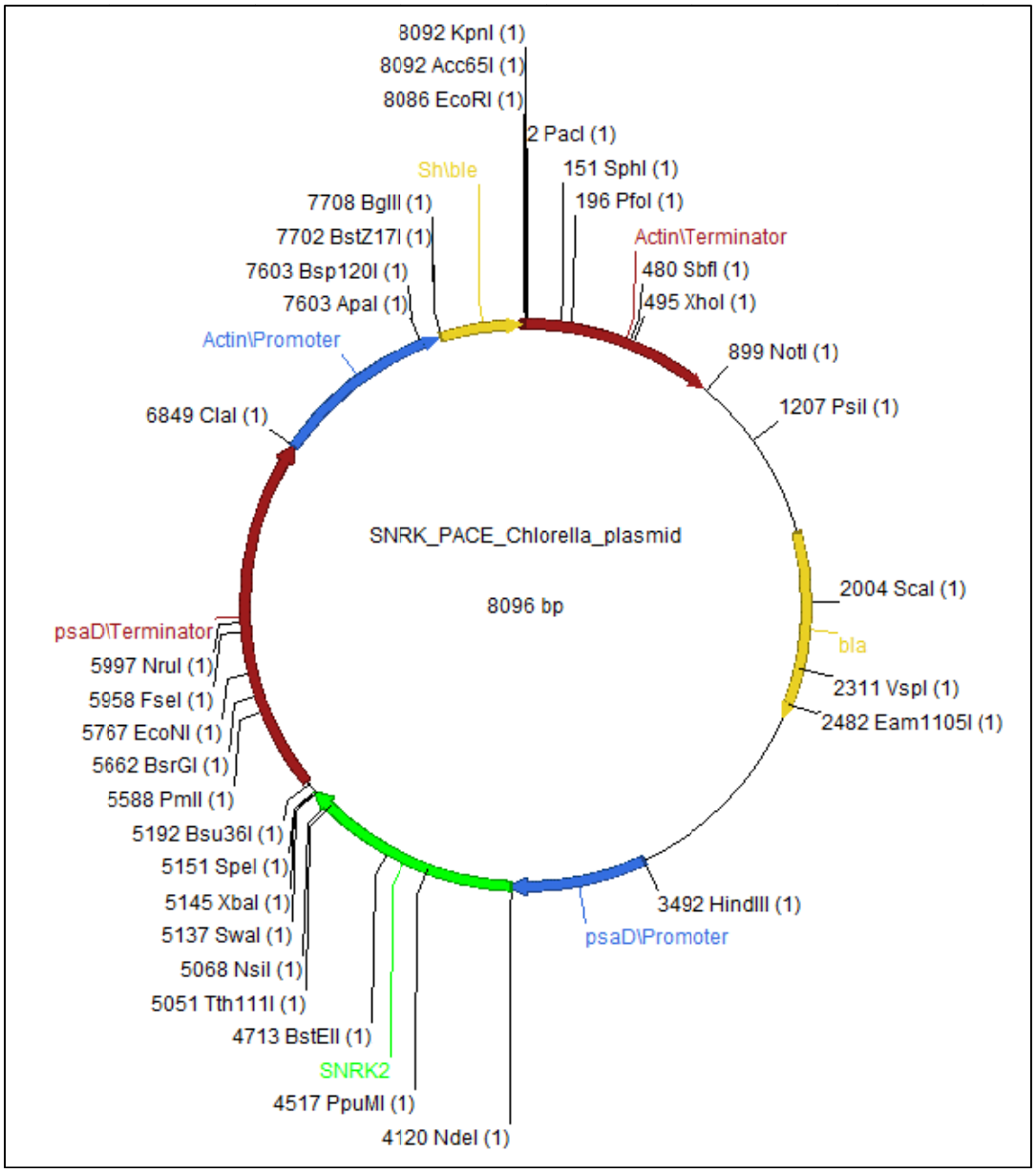

Figure 3. The SNRK_PACE_Chlorella_plasmid

As noted by the submitters, the critical components of this vector are the *psaD* promoter sequence, the modified intergeneric gene coding sequence (*SNRK2*), the extended *psaD* terminator, as well as the selection marker, which should integrate as an actin promoter, the zeocin resistance gene (*ble*) coding sequence, and the actin terminator.

This vector (SNRK_PACE_Chlorella_plasmid) was linearized with the restriction enzyme ScaI before being introduced into the recipient microorganisms *C. sorokiniana* DOE1412 via electroporation (depicted in Figure 4). This restriction digest also cleaved the ampicillin resistance gene *bla* in half (at position 2004), so that a functional beta-lactamase is not expected to be expressed. Therefore the final submission strain PACE_Cs1412_SNRK2 has neither a full nor functional ampicillin gene, and hence, will not be resistant to that antibiotic.

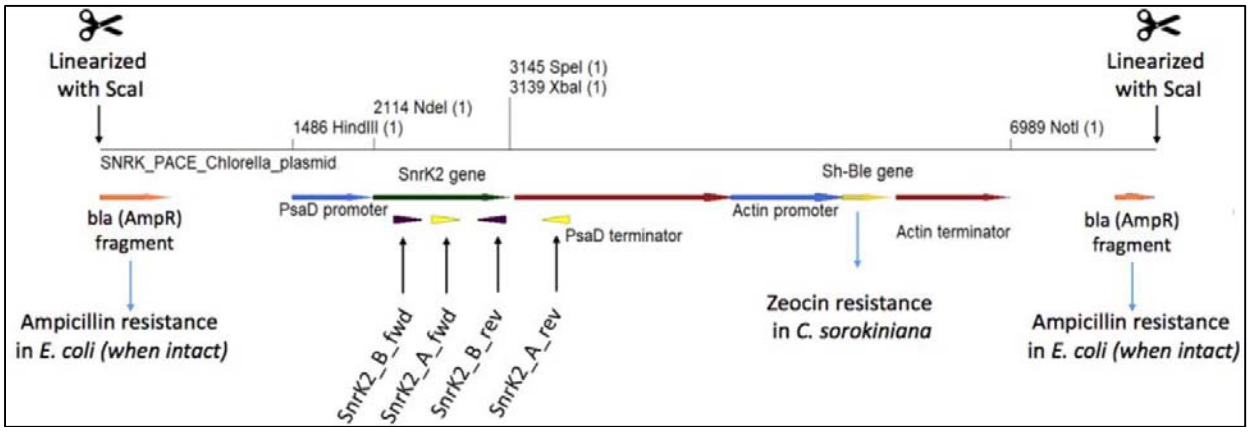

Figure 4. Depiction the SNRK_PACE_Chlorella_plasmid (Figure 3) when linearized by ScaI.

For this TERA's submission strain, it is expected at least one copy (personal communication) of the intergeneric construct containing the foreign DNA is integrated in the nuclear genome and expressed in the cytoplasm. This is because the zeocin marker gene, Sh *ble*, is reported not to be effective if expressed in the chloroplast (Fischer and Rochaix, 2001). In addition, as the *SNRK2* and marker gene (Sh *ble*) are part of the same integrated DNA fragment, they are expected to be located together and expressed in the same algal cellular compartment. The submitters are in the process of sequencing the submission strain to determine specifics, including copy number and integration sites.

VIII. CONSTRUCT HAZARD ANALYSIS

The potential hazards posed by the genetic modifications and the potential for horizontal and vertical gene transfer of the introduced genetic material were analyzed by Nguyen (2018a).

A. Introduced Genes

1. Sucrose non-fermenting related kinase 2 (*SNRK2*) gene

The sucrose non-fermenting (snf) related kinase 2 gene, *SNRK2*, is part of the serine/threonine kinases (Kertesz et al., 2002) and plays a key role in sugar metabolism in plant and animal kingdoms and controls multiple growth and metabolic processes.

Members of the sucrose non-fermenting related kinase Group2 (SNRK2) subclasses have been studied primarily in plants (e.g., *Arabidopsis*) and are implicated in both direct and indirect abscisic acid (ABA) response pathways dealing with environmental stress-signaling (Holappa et al., 2017; Todaka et al., 2015). Although the ABA signaling pathways have been extensively studied in plants, knowledge of their roles in algae and other lower photosynthetic species (e.g., cyanobacteria and lichens) remain limited. ABA synthesis in algae however is known to be induced by environmental stressors like drought or salt stress (Hartung, 2010). It has been shown that all SNRK2 subclasses are well conserved among higher plants, yet SNRK2s in algae (e.g., *Chlamydomonas*) have been classified as having distinct sequences from those found in higher plants (Hauser et al., 2011).

When overexpressed in *Arabidopsis*, *SNRK2* conferred increased sucrose synthesis, starch synthesis, and leaf growth (Zheng et al., 2010). The SNRKs have also been detected in almost all streptophyte algae (de Vries et al., 2018), and implicated with cold stress adaptation for the alga *C. reinhardtii* (Valledor et al., 2013). Streptophyte algae are a small group of freshwater algae ranging from scaly, unicellular flagellates (*Mesostigma*) to complex, filamentous thalli with branching, cell differentiation and apical growth (Charales). Streptophyte algae and embryophytes form the division Streptophyta, whereas the remaining green algae are classified as Chlorophyta (Becker and Marin, 2009).

The submitter expected the overexpression of *SNRK2* would improve starch accumulation and growth in *Chlorella* cells. In preliminary experiments, the submitters concluded that compared to wild-type *C. sorokiniana* 1412, the submission microorganism PACE_Cs1412_SNRK2 showed improved photosynthetic efficiency and growth (biomass).

2. Zeocin resistance (*ble*) gene

The submission strain PACE_Cs1412_SNRK2 also contains the *Streptoalloteichus hindustanus* Sh *ble* gene that encodes resistance to the antibiotic zeocin. The *ble* gene also encodes resistance to other members of the glycopeptide antibiotic bleomycin family, bleomycins, phleomycins, and tallysomycins. Zeocin™ is the commercial name for a special formulation of phleomycin D1 (Ceozin) (http://www.genaxxon.com/docs/pdf/zeocin_data.pdf). According to this website, there is no cross resistance of zeocin with other antibiotics such as G418 (neomycin/geneticin), hygromycin B, blasticidin S, or puromycin.

Zeocin belongs to the bleomycin (BLM) family of antibiotics, which have been widely used as chemotherapeutic agents for the treatment of skin, head and neck carcinomas. BLMs damage DNA directly, and some iron complexed BLMs have been reported to cause sequence-specific DNA cleavage in the presence of oxygen (Gatignol et al., 1988). BLMs are harmful to any cells that come in contact with them, including the BLM-producing organisms. To protect themselves, BLM-producing organisms also produce proteins that can modify and sequester BLMs (Miyazaki et al., 2009).

Zeocin has no clinical uses in humans as an antibiotic to treat bacterial infections. Bleomycin and phleomycin are also not used as antibiotics to treat bacterial infections in humans. Thus, there is no concern for comprising the therapeutic value of antibiotics in treating infections if the *ble* gene were to be transferred to pathogens in the environment (Salazar, 2018).

Although BLMs have not been used as antibacterial agents, many clinically isolated strains of methicillin-resistant *Staphylococcus aureus* (MRSA) produce BLM-binding proteins that sequester these antibiotics, leading these strains to be resistant to BLMs at high levels. Other BLM-binding proteins have been found in *Escherichia coli* (Miyazaki et al., 2009). According to van Peer et al. (2009) and Gatignol et al. (1988), phleomycin and bleomycins are commonly used as selection markers for transformations in algae, protists, fungi, and animals.

As previously mentioned, in the development of the submission strain, the PACE_*Chlorella*_Zeocin_Plasmid that was used has a resistance gene to the clinically important antibiotic ampicillin. However, the SNRK_PACE_Chlorella_plasmid was linearized before transformation by a single restriction enzyme digest that disrupts the AmpR gene (*bla*), therefore neither a full or functional *bla* encoding sequence remains in the genome of the submission strain.

B. Potential for Horizontal and Vertical Gene Transfer

The intergeneric genes are expected to be stably integrated into the nuclear genome. The stability of the DNA can be assessed by PCR and qPCR after multiple generations of growth. The submission microorganism has been reported to have had no loss of the introduced DNA for two years after the initial transformation event, as observed by the submitters.

As mentioned earlier, the submitters previously reported that under strict laboratory conditions they have been able to induce the production of haploid gametes in intermediate transgenic strains, which allowed them to do backcrossing (to avoid any reversions to a wildtype genetic background) to arrive at the final strain for their last TERA submission (R-17-0002). However, backcrossing was not done for this submission strain, PACE_Cs1412_SNRK2, since *SNRK2* overexpression has been observed by the submitters to be conserved in the transgenic lines for two years via PCR/RT-PCR. As a result, backcrossing was considered to be unnecessary in this specific case for the stability of *SNRK2* overexpression in PACE_Cs1412_SNRK2.

PubMed searches carried out by the submitter on *C. sorokiniana** gene transfer* genetic exchange* did not find any articles on gene exchange of the recipient microorganism in natural populations. Prior to initiating the field trial experiments, the submitter will sample soil from the field test site to determine what organisms may be present in the microbial community within the soil. The submitter will also perform a similar analysis on the local surface water from the canal system to the east of the ASU Polytechnic campus.

Horizontal gene transfer among bacteria is widespread and is responsible for the acquisition of new traits such as antibiotic resistance. Not nearly as much is known regarding horizontal gene transfer in eukaryotes. It has been thought that the barriers to horizontal gene transfer in eukaryotic organisms greatly exceeds that noted for bacterial species (Raymond and Blakenship, 2003). However, from evolutionary analyses, horizontal gene transfer in eukaryotes is known to have occurred. For example, it has been hypothesized that in evolutionary times, a primary event in the creation of plants was the endosymbiotic engulfment and retention of a cyanobacterial species. This event gave rise to the photosynthetic plastid in the common ancestor of the Plantae, such as red and green algae and higher plants (Chan et al., 2012). Likewise, the mitochondria apparently arose from the endosymbiosis and subsequent genetic integration of an alpha-proteobacterium (Keeling and Palmer, 2008; Timmis et al., 2004). In addition, investigations of the *Chlorella* genome, specifically *Chlorella variabilis*, suggest the ability for *Chlorella* to produce chitinous cell walls as a result of genetic material uptake from algal viruses, prokaryotes, and fungi (Blanc et al., 2010). Eckardt et al. (2010) hypothesized that the *Chlorella* chitin metabolism genes could have been acquired via horizontal gene transfer from viruses. There are other episodes of lateral gene transfer in eukaryotes, such as those that have occurred from phagocytosis of the alga *Vaucheria litorea* by the sea slug *Elysia chlorotica* (Rumpho et al., 2008). The photosynthetic sea slug maintains the algal plastids which continue to photosynthesize for months within the slug.

Very little is known about horizontal gene transfer from one algal species to another. However, there is evolutionary evidence for horizontal gene transfer in algae. Archibald et al. (2003) found that of the 78 plastid-targeted proteins in the chlorarachniophyte alga *Bigelowiella natans*, approximately 21% of them had probably been acquired from other organisms including streptophyte algae, red algae (or algae with red algal endosymbionts), and bacteria. However, in the green alga *Chlamydomonas*

reinhardtii, the homologous genes did not show any evidence of lateral gene transfer. It was suggested that this may be because this green alga is thought to be solely autotrophic whereas the *Bigelowiella* is both photosynthetic and phagotrophic. Another instance of potential lateral gene transfer having occurred in algae is the work presented by Raymond and Kim (2012). They found the presence of ice-binding proteins in sea ice diatoms that apparently are essential for their survival in the ice. These protein genes were completely incongruent with algal phylogeny, and the best matches were all bacterial genes. Like bacterial genes, they did not contain introns. There is one example of horizontal gene transfer from an alga to its DNA virus. By phylogenetic analysis, Monier et al. (2009) demonstrated the transfer of an entire metabolic pathway consisting of seven genes involved in sphingolipid biosynthesis from the eukaryotic alga *Emiliania huxleyi* to its large DNA virus known as EhV.

There is no information in the literature on horizontal gene transfer specifically associated with *Chlorella*. Acquisition of the *SNRK2* gene from PACE_Cs1412_SNRK2 by other algae in the environment could provide them with increased growth rates and photosynthetic efficiency. However, since horizontal gene transfer from *Chlorella* to other algae have not been specifically documented, this is considered unlikely to occur.

There is a possibility for vertical gene transfer through mating with wild *C. sorokiniana* strains. Production of sexual cells, gametes, has been observed for the green alga *Scenedesmus obliquus* (Trainor, 1998). However, it was only under extremely specialized laboratory conditions that gametogenesis could be induced (Trainor, 1998). Likewise, the submitter also notes that although they have previously seen rare indications of haploids and sexual reproduction by microscopy in a closely related strain (*C. sorokiniana* 1228*)*, they generally observe cell division to occur via non-motile asexual autospores, which is consistent with published literature where *C. sorokiniana* is reported to be asexual (Cazzaniga et al., 2014). Furthermore, *C. sorokiniana* DOE1412, the recipient strain for this application has not been reported to sexually reproduce in the lab, or in any published work. So it is unlikely that sexual reproduction would contribute to gene transfer of the *SNRK2* gene to other algae.

IX. POTENTIAL HUMAN HEALTH HAZARDS OF THE RECIPIENT MICROORGANISM

The potential human health hazards of the recipient *C. sorokiniana* strain to the general population and to potentially exposed and susceptible subpopulations have been evaluated (Salazar, 2018).

1. General Population

A. Pathogenicity

C. sorokiniana is capable of growth at human body temperature (37°C) as it is one of the most heat tolerant species in the genus. However, there is no evidence in the literature that the species *C. sorokiniana* causes infections in humans. According to the submission, the American Type Culture Collection (ATCC) lists *C. sorokiniana,* along with 22 other strains of the genus *Chlorella,* as Biosafety Level 1 (BL1) organisms based upon the fact that these organisms are not known to cause disease in healthy humans.

However, in extremely rare cases, *Chlorella* has caused infections in humans and other animals. Chlorellosis is the name of this infection by *Chlorella*. It has occurred in limited numbers in sheep and cattle, rarely in humans, and in single cases in a dog, gazelle, beaver, camel, and fish (as summarized by Hart et al., 2014). Animals are infected by exposure of open wounds to contaminated water. In mammals, this disease ranges from localized cutaneous infection, lymph node infection, or dissemination to multiple organs. However, in humans, the three reported cases were cutaneous infections (Jones et al., 1983; Yu et al., 2009; Hart et al., 2014). Chlorellosis in humans is extremely rare. Although *Chlorella* is prevalent globally in fresh water lakes and rivers, in marine waters, and in soil, there have been just three reported cases. Another green alga, *Prototheca* that has been shown to infect humans at a higher rate (more than 100 cases have been reported). However, infection by *Prototheca* is also rare as this alga is widespread in the environment and thus, humans are highly exposed.

The first case of chlorellosis in humans was described by Jones et al. (1983) where a 30-year-old woman developed a persistent infection of a healing operative wound on her right foot after possible contamination by river water while canoeing. The wound was debrided two months later and the infection then treated with antibiotics and wound irrigation. The infection was persistent and healed completely after 10 months.

The second case of *Chlorella* infection was an external infection found in the gangrene tissue from the right foot of a diabetic 59-year-old female (Yu et al., 2009). The *Chlorella* isolate was thought to be *C. saccharophila,* a *Chlorella* strain that uses glucose as a sole carbon source, grows at pH 2-3, and grows at temperatures up to 30°C. The authors stated the strain "could not grow at 37°C in light or darkness. The results suggest that this strain may not normally invade tissues, but becomes established and grows on previously infected tissues of external body extremities where the temperature is somewhat lower than normal body temperature."

The most recent case of chlorellosis was reported in Australia in a 30-year-old man in a knee wound contaminated with fresh water dam water (Hart et al., 2014). He developed a *Chlorella* and *Aeromonas hydrophila* infection within two days of exposure and the infection was aggressive and required debridement, negative pressure wound dressings, and antibiotics. However, the wound had healed by the third week with no further complications.

Overall, chlorellosis in humans is extremely rare as there have been just the three reported cases mentioned above, even when the alga *Chlorella* is known to be widespread. Even with more than 100 cases reported of human infections from the alga, *Prototheca*, it is also still considered rare since *Prototheca* is widespread in the environment, where humans are constantly exposed to. The fact that such few *Chlorella* infections have been reported, and considering that *Chlorella* is a prevalent alga in fresh water, marine waters, and in soils where humans are frequently exposed to the alga, implies that chlorellosis is quite rare.

B. Toxicity

According to the submission, there are no reports in the literature that any *Chlorella* species, including *C. sorokiniana,* synthesizes or secrets phycotoxins. Recently, toxicity studies with *C. sorokiniana* revealed no toxicity either *in vitro* cytotoxicity assays or in subchronic toxicity studies in rats (Himuro et al., 2017).

The lack of toxin production by *Chlorella* allows it to be used as a popular human nutritional supplement. In addition, *Chlorella* extracts are used in skin care products. *Chlorella* has been proposed as a protein supplement for human consumption (Becker, 2007). *Chlorella* sp. are generally regarded as safe (GRAS) for human consumption. *Chlorella* sp. and *C. protothecoides* flours have GRAS status (GRN 000330; GRN 000519) with the Food and Drug Administration (FDA). In humans, *Chlorella* sp. supplements have shown beneficial effects including improved immune responses, improved healing of the small intestine epithelium, antioxidant action and even anti-tumoral effects (Ramirez-Romero et al., 2010). *C. vulgaris* has been promoted as a prevention of anti-inflammatory responses (Hasegawa et al., 1999). Morin et al. (1980) have shown inhibitory effects of the unicellular alga *Chlorella* against murine sarcomas. *C. sorokiniana* was also found to activate dendritic cells resulting in T cells activation (Chou et al., 2012). Lastly, *C. sorokiniana* extract was shown to increase short term memory in rats (Morgese et al., 2016). A company called Taiwan Chlorella has been growing *Chlorella* for nutritional supplements since 1964, and specifically grows *Chlorella sorokiniana* strain UTEX1230 for some of its *Chlorella* nutritional supplements (http://www.taiwanchlorella.com/chlorella01.php).

There is one study in the literature that reported cytotoxicity of algal dietary supplements consisting of a mixture of *Chlorella* sp. and the collective cell biomass from two cyanobacteria, *Arthrospira platensis* and *A. maxima* commonly referred to as *Spirulina* (Heussner et al., 2012). They found extracts from 13 commercially available products sold in Germany were cytotoxic in the A549 cell line with the *Spirulina* being more potent than *Chlorella*. This toxicity, however, was due to contamination of the cyanobacterial and algal cultures by microcystin, a potent toxin produced by the cyanobacterium *Microcystis.* The toxicity was not due to the *Chlorella* or *Spirulina.*

C. Allergenicity

Allergy is the result of a marked increase in reactivity and responsiveness of an immune response to a protein or a low molecular weight compound combined with a larger “self” molecule. However, recent research suggests that not every protein is allergenic (Radauer et al., 2008).

Humans may be routinely exposed to high numbers of algal cells on a daily basis through respiration in both indoor and outdoor environments. Algae and cyanobacteria usually constitute a minority of airborne bioaerosols compared to fungi, pollen, and bacteria; however, in certain cases the quantity of airborne algal particles can far exceed that of fungi spores and pollen grains (McGovern et al., 1965). Brown et al. (1964) found over 3000 algae/m^3 in samples taken from a car moving through a dust cloud in Texas. Schlichting (1969) found < 8 algal cells/ft^3 in air sampled in Texas, Michigan, and off the North Carolina coast and calculated that breathing 240 algae cells per hour was possible for a maximum daily uptake of 2880 algal and cyanobacterial cells. In a summary of the existing literature on airborne algae during the years 1910 - 1968, a total of 187 taxa of algae and protozoa were found. Several species of *Chlorella* were sampled directly from the air including *C. ellipsoidea, C. pyrenoidosa, C. vulgaris,* and *Chlorella* sp. (Schlichting, 1969). Bernstein and Safferman (1970) also found 18 different genera of algae in house dust collected from 41 homes of which *Chlorella* was the most frequently encountered algae, followed by *Chlorococcum, Schizothrix, Planktosphaeria, Chlamydomonas, and Anabaena.*

There is evidence from human studies that *Chlorella* can induce hypersensitivity responses in some individuals. Tiberg et al. (1995) tested Swedish children for allergy to *Chlorella* using three methods: the radioallergosorbent test (RAST), skin prick tests (SPTs), and conjunctival provocation tests (CPT). These tests detect specific IgE antibodies to determine whether a subject is sensitized to the substance. No *Chlorella*-specific IgE antibodies were found in the sera from the 94 children from the general population

(group 1 – no allergy symptoms). In a group of children that had been referred to an outpatient pediatric allergy clinic (group 2), nine of the 129 children had positive wheal reactions with the *Chlorella* extract in SPTs. Sera from seven of these children with positive SPTs results were available for analysis of IgE antibodies. Two of the seven were positive for IgE-specific antibodies to Chlorella. Seven of 23 mold-sensitive children (group 3) had positive SPTs to *Chlorella.* Six patients with SPT positive results and two of the 16 patients with negative SPT results had positive RAST results. All patients with positive SPT results showed some reaction in CPTs with *Chlorella* extract (5 mg dry weight/ml). These data demonstrate that only children that are sensitized to many common allergens also were sensitized to *Chlorella* and no specific symptoms related to *Chlorella* sensitization were observed. These data suggest that *Chlorella* is a weak allergen.

Similarly, Bernstein and Safferman (1966) tested two species of *Chlorella, C. vulgaris* and *C. pyrenoidosa,* two species of *Chlorococcum, C. botryoides* and *C. macrostigmatum*, *Scenedesmus basilensis,* and *Ankistrodesmus falcatus* var. *acicularis* for their potential to elicit cutaneous reactions in atopic patients, i.e., those with a genetic predisposition for developing allergic hypersensitivity reactions. They found that of 79 atopic patients tested with algal extracts, 47 also gave positive skin reactions while non-atopic individuals did not show positive skin reactions. Additional tests with *C. vulgaris* for bronchial mucosa tests resulted in clinical wheezing. Interpretation of this study is greatly limited by lack of antigen quantification or understanding of the cellular and molecular mechanisms involved and small sample sizes.

There is a single report of occupational asthma in a pharmacist induced by exposure to a fine dust powder of *Chlorella* while making chlorella tablets for human consumption (Ng et al., 1994). It was suggested that the causative agent in this chlorella-induced asthma was pheophorbide-a, which is a breakdown product of chlorophyll, and its ester, or some other protein component. Pheophorbide-a and its ester are formed by the reaction of the chlorophyllase enzyme during the drying process of the moist *Chlorella* cells with heated air at 90°C. Given that the hypersensitivity response was induced by fine, dry dust created by high heat, the relevance of this report of occupational asthma to exposures of live moist *Chlorella* cells in bioaerosols during this field test is questionable.

The database Allergome lists *Chlorella* as an allergen, however the WHO/IUIS Allergen Nomenclature database (Allergen Nomenclature (IUIS); http://www.allergen.org) does not. Based on a review of outdoor allergens, Burge and Rogers (2000) stated that algae do not seem to be a source of major outdoor allergens.

The submitters state that none of their workers have responded to *C. sorokiniana* adversely despite years of cultivation in both closed reactors and open ponds. Although they have only been growing *C. sorokiniana* outdoors for one year, they have been growing various strains of *Chlorella* for more than 10 years at the site with no allergenicity symptoms due to this alga genus.

D. Other Effects

Chlorella has also been reported to cause photosensitization, which is development of abnormally heightened reactivity of skin or eyes to sunlight, in those who took *Chlorella* as a dietary supplement (Jitsukawa et al., 1984). In addition, protein components of *Chlorella* such as a breakdown product of chlorophyll, pheophorbide-a and its ester that are recognized as photosensitizers may contribute to adverse reaction in the kidney (Yim et al., 2007). However, this photosensitization resulted from ingestion of algae which is not relevant to exposures in this TERA field test.

2. Potentially Exposed and Susceptible Subpopulations

Potentially exposed individuals are workers at the AzCATI facility. Susceptible subpopulations that warrant consideration differ whether in relation to potential pathogenicity or allergenicity of *Chlorella.* In terms of pathogenicity, susceptible subpopulations would include those whose immune systems are not fully competent such as the young, the elderly, malnourished individuals, and those with pre-existing disease or on immunosuppressive therapies. Susceptible populations for allergenicity concerns are atopic individuals which are those with a genetic predisposition toward developing hypersensitivity reactions to environmental antigens.

A. Pathogenicity

The recipient microorganism *C. sorokiniana* is generally characterized as one of the most heat tolerant species in the genus and strains of the species are thus capable of growth at human body temperature (37°C). According to the submission, the recipient strain *C. sorokiniana* DOE1412 grows well at temperatures lower than human body temperature, from approximately 15 - 32°C with an optimum around 28°C. It can tolerate short duration excursions to higher temperatures, up to 40°C although with a much-reduced growth rate.

As previously mentioned, *C. sorokiniana* has not been reported as causing any infections in humans. However, there are three reports of *Chlorella* sp. infections in humans originating in open wounds after exposure to contaminated water. Chlorellosis is extremely rare even though humans are routinely exposed to *Chlorella* as it ubiquitous in the environment in fresh waters, marine waters, and in soils, and even found in the indoor environment in house dust. Thus, there is little concern even for those with not fully competent immune systems as they too are routinely exposed to *Chlorella* sp. Dermal contact of workers to the alga in the open miniponds is not expected as workers will be wearing personal protective equipment required by ASU EH&S regulations (e.g., gloves, lab coats, long pants, closed-toe shoes) when handling the algae.

B. Toxicity

In regards to toxicity, there is low concern for potentially exposed or susceptible subpopulations as well as the general population as *Chlorella* is not known to produce any phycotoxins.

The introduced genetic material, the *SNRK2* and Sh *ble* genes also do not pose any toxicity concerns. As mentioned above, the SNRK2 enzyme is part of the serine/threonine kinases (Kertesz et al., 2002) and plays a key role in sugar metabolism in plant and animal kingdoms and controls multiple growth and metabolic processes. This group of kinases (SNRKs) are widely found in higher plants, algae, bacteria, and mammals including humans (Kertesz et al., 2002).

C. Allergenicity

There may be some concern for allergenicity with potentially exposed and susceptible subpopulations if any workers are atopic individuals that are prone to developing hypersensitivity reactions even though *Chlorella* has been characterized as being a “weak” allergen (Tiberg, 1995). Bioaerosols containing algal cells are expected to be generated during the growth of the algae in open raceway miniponds so some inhalation of the submission strain PACE_Cs1412_SNRK2 is expected. The general human population

does not appear to suffer allergenicity symptoms from exposure to *Chlorella* since *Chlorella* is ubiquitous in the environment in fresh water, marine waters, and soils, and even occurs in house dust so humans routinely inhale *Chlorella* cells. As previously mentioned, the submission states that they have been growing naturally occurring *Chlorella* strains for 10 years with none of the workers at the AzCATI facility suffering allergenicity symptoms. Based on a review of outdoor allergens, algae do not seem to be a source of major outdoor allergens (Burge and Rogers, 2000). It is unlikely that atopic individuals would choose to work with algae given their predisposition to developing hypersensitivity reactions. However, if atopic individuals work at the facility, allergenicity symptoms could be alleviated by the use of respirators (APF50 respirators with P100 filters that removes 99.97% of exposure to microorganisms).

X. POTENTIAL HUMAN HEALTH HAZARDS OF THE SUBMISSION MICROORGANISM

The potential human health hazards of the submission microorganism PACE_Cs1412_SNRK2 to the general population and to potentially exposed and susceptible subpopulations have been evaluated (Salazar, 2018).

1. General Population

The concern for pathogenicity or toxicity associated with the introduced genes is low. As described by the submitters and the Construct Hazard Analysis (Nguyen, 2018a), the introduced intergeneric DNA sequences for the sucrose non-fermenting related kinase (*SNRK2*) gene is part of the serine/threonine kinases and plays a key role in sugar metabolism in plant and animal kingdoms and controls multiple growth and metabolic processes and is not expected to contribute pathogenicity or toxicity to the parental alga *C. sorokiniana*.

The introduced *ble* gene used as a selection marker which encodes resistance to the antibiotics zeocin, phleomycin, and bleomycin does not pose human health concerns. As previously mentioned, zeocin is a laboratory chemical that is not approved for use in human or animal medicine. Phleomycin and bleomycin are used for their anti-cancer properties, but they are not used to treat bacterial infections in humans. Thus, the general concern for loss of the therapeutic value of an antibiotic resulting from horizontal gene transfer to pathogens in the environment whose infections are treated with that antibiotic does not exist.

The submitter also conducted an allergen search for both the Sh *ble* and *SNRK2* genes. A FASTA search against the allergenonline.com database yielded no hits for Sh *ble* indicating that it is not allergenic. With *SNRK2*, the submitters identified the open reading frames (ORFs) and conducted a FASTA search for each. This search yielded no hits above 35% identity for the 80-mer sliding window and the 8-mer exact match searches yielded no hits. The 8-mer search identifies any 8-amino-acid contiguous matches, while the more conservative 80-mer search identifies any 80-amino-acid stretches with >35% match (Goodman, 2006). Again, both Sh *ble* and *SNRK2* genes encode for intracellular proteins so there is no expected exposure to the proteins themselves by the respiratory route. Therefore, the concern for allergenicity associated with the introduced genes is low.

2. Potentially Exposed and Susceptible Subpopulations

The genetic modifications of the recipient to make the submission strain PACE_Cs1412_SNRK2 strain do not pose adverse human health effects to potentially exposed and susceptible subpopulations just as they do not to the general human population. The SNRK2 enzyme does not pose pathogenicity, toxicity or allergenicity concerns to humans.

The zeocin resistance gene also does not pose any additional concerns to potentially exposed and susceptible subpopulations relative to the recipient strain.

XI. POTENTIAL ECOLOGICAL HAZARDS OF THE RECIPIENT MICROORGANISM

The potential ecological hazards of the recipient and the submission microorganisms have been evaluated by Nguyen (2018b).

Three genera of green algae, *Chlorella, Chlamydomonas,* and *Scenedesmus,* are the dominant green algae in many aquatic habitats and are frequently isolated from marine, fresh water, soils and air samples, as they can tolerate a wide range of environmental conditions (Trainor, 1998). *Chlorella* is a simple airborne microalga, present in terrestrial and aquatic habitats, whose minute cell size and resistance against environmental stress allows for long-distance dispersal (Hodac et al., 2016). *Chlorella* is an aerophilous algae (found in air), a type of algae shown to have better adaptation and growth responses compared to their solely soil and aquatic counterparts (Sharma et al., 2007).

Chlorella is resistant against a number of environmental stressors due to its metabolic versatility, and thus, is able to cope with shortages of nutrients and water. This genus has a high tolerance to temperature and can easily live in both terrestrial and aquatic ecosystems. Members of the genus *Chlorella* are found in freshwater natural and artificial water habitats throughout the world (Trainor, 1998) and some species can even thrive in polar regions and hot deserts (Hodac et al., 2016). *Chlorella* have been reported from nearly all soil types, including desert soil crusts. *Chlorella* was one of the most common genera found across 4 of 7 different biomes sampled across the Namibian-Angola border (Budel et al., 2009), humic tropical soils in India, biofilms covering natural and artificial subaerial substrates, and can live in different soils such as polar desert soils in Antarctica and the Arctic (Hodac et al., 2016). They can be also grown in wastewater and used for the removal of metals (de-Bashan et al., 2008). Phylogenetic analysis (using SSU and ITS2 rDNA sequencing) has shown their polar, temperate and tropical distribution, in addition to demonstrating that even polar isolates are closely related to temperate ones (Hodac et al., 2016). Based on sequence similarities Hodac et al. (2016) concluded that *Chlorella* might be capable of intercontinental dispersal; however, they acknowledge that their actual distributions may exhibit biogeographical patterns but requires further research. Although most *Chlorella* species are naturally free-living, some are known photosynthetic symbionts, such as one species known to be a symbiont of the unicellar protozoa *Paramecium bursaria* (Blanc et al., 2010).

Although some genera in the class Trebouxiophyceae can cause harmful algal blooms (HABs) such as the genus Tetraspora, *Chlorella* are not associated with harmful algal blooms. The genus is not listed as a harmful species, including in UNESCO's list of harmful micro algae (http://www.marinespecies.org/hab/

visited October 2018). The genus thrives in higher temperatures than other common species with moderate nutrient loaded environments; so it is known to bloom later in the year (Elliot et al., 2006; Cordero et al., 2011). Although *Chlorella* has the potential of producing dense blooms, to date there is no available literature showing that *Chlorella* blooms have caused any adverse effects (Ryther, 1954). The only references that cite a *Chlorella* bloom event (Pan et al., 2011; Li and Pan, 2013) are based on erroneous interpretation of the paper by Ryther (1954) that mentions *Chlorella.* However, the observed decimation of the oyster industry on Long Island that was attributed to eutrophication stimulated by duck farm effluents Ryther (1954) led to blooms of *Nannochloris atomus* and *Stichoccocus sp.*, not *Chlorella.* So, to date, there has been no recorded HAB event associated with *Chlorella sp.*

Some *Chlorella sp.* produce a substance known as chlorellin which is an antibiotic-like substance that can inhibit its own growth and that of Gram positive and Gram negative bacteria (Pratt, 1944). Older literature has demonstrated that *Chlorella* can produce substances that are inhibitory to the growth of other algae such as *Nitzschia frustulum* (Rice, 1949). These experiments simply exposed competing algae to the exudates of *Chlorella sp.* and did not characterized the specific molecule(s) associated with the inhibitory effect. It is possible that *Chlorella* may be able to inhibit the growth of other organisms by producing chlorellin or some other inhibitory molecule. However, the ability of microorganisms to produce substances inhibitory to other microorganisms is commonplace. Many microorganisms, including bacteria and fungi are capable of producing antibiotics, antifungal compounds, and other inhibitory substances which are involved in the complex interactions among microorganisms in the environment (Abrudan et al., 2015).

1. Potential Effects of Chlorella sp. on Terrestrial Mammals

As mentioned above in the human health section, *Chlorella* is capable of causing infections in mammals including humans known as chlorellosis. Infection of tissues by *Chlorella* has been reported in a limited number of cases of in mammals including sheep (both adults and lambs), cattle, dogs, and in single cases in a gazelle, a camel, a beaver, and a fish (Cordy, 1973; Kaplan et al., 1983; Le Net et al., 1993; Philby, 2001; Haenichen et al., 2002; Quigley, et al., 2009; Ramirez-Romero et al., 2010; Hart et al., 2014). Documented cases of mammalian chlorellosis are extremely rare and are typically opportunistic infections resulting from contamination of wounds or dissemination from the gastrointestinal tract following oral ingestion of stagnant water or sewage-contaminated water (Kaplan et al., 1983; Zakia et al., 1989; Philby et al., 2001; Haenichen et al., 2002; Ramirez-Romero et al., 2010). Effects of chlorellosis in terrestrial mammals include the formation of lesions in the skin, liver, lungs and lymph systems accompanied by a characteristically green discoloration of the affected organs (Ramirez-Romero et al., 2010). There is limited information available to characterize chlorellosis infections in terrestrial wildlife. However, chlorellosis in animals, as in humans, is very rare as only a few cases have been reported when *Chlorella* is ubiquitous in aquatic and terrestrial environments, thus animals are routinely exposed to it through dermal contact, inhalation, and ingestion in drinking water.

2. Potential Effects on Plants

There is no information in the literature that suggests any negative effects of *Chlorella* on aquatic or terrestrial plants.

XII. POTENTIAL ECOLOGICAL HAZARDS OF THE SUBMISSION MICROORGANISM

As discussed in the Ecological Hazard Assessment (Nguyen, 2018b), the *SNRK2* gene is expected to and was shown by the submitters to help the proposed strain, PACE_Cs1412_SNRK2 have better growth and photosynthetic efficiency than wild-type recipient *C. sorokiniana* DOE1412. It has been reported in the literature that Arabidopsis *SNRK2*, when overexpressed, conferred increased sucrose synthesis, starch synthesis, and leaf growth (Zheng et al., 2010). The SNRK group of kinases have also been detected in almost all streptophyte algae (de Vries et al., 2018), and implicated with cold stress adaptation for the alga *C. reinhardtii* (Valledor et al., 2013). In preliminary experiments, the submitters report a 26-30% increase in growth (low to high light) (see Figure 5), along with a 21% increase in total carbohydrate accumulation in PACE_Cs1412_SNRK2 compared to wild-type *C. sorokiniana* 1412 (R-18-0001).

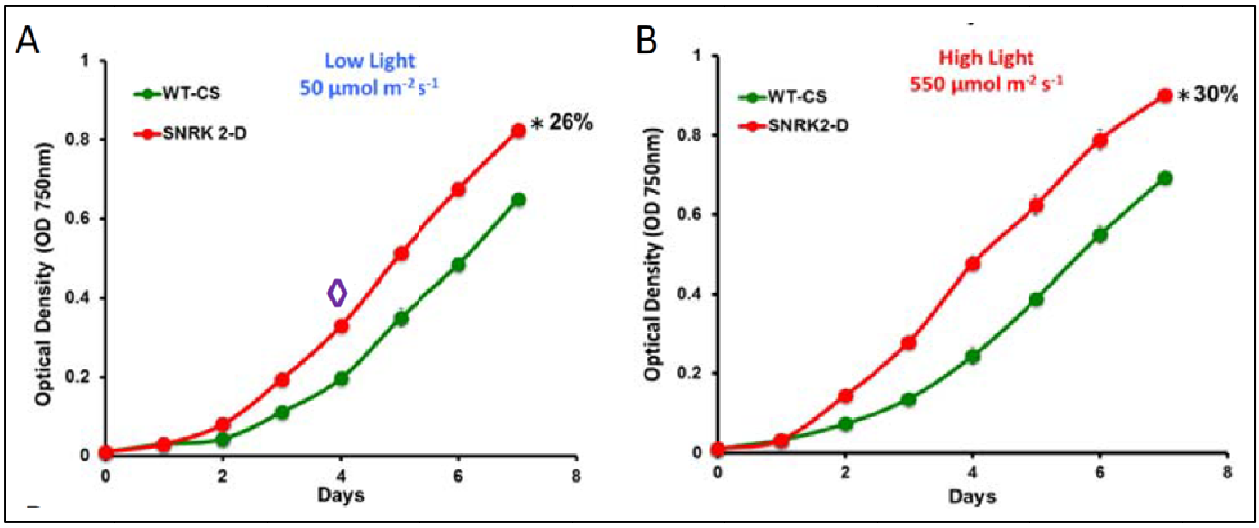

Figure 5. Photoheterotrophic growth of SNRK2 vs. wild type *C. sorokiniana* (A) Low light intensity. (B) High light intensity. (Figure 26 of TERA R-18-0001)

The growth characteristics of *Chlorella sorokiniana* has been extensively studied in literature due to its high performance in various factors (biomass, lipids, growth rate, and temperature tolerance) (Sayre et al., 2015). Many indoor/outdoor growth studies have been performed with this strain in attempts to optimize its productivity in various biotechnology fields. The genetic modifications presented for this submission enhances the growth and biomass accumulation of the submission microorganism, which can be viewed as increase in its competitive advantage in the environment as it will consume more nutrients at a faster rate than that of the wild type recipient.

However, the survival characteristics are not expected to drastically change from the wild type recipient to the submission strain. Although the introduced genetic material does enable faster growth, it does not enable PACE_Cs1412_SNRK2 to survive in environments not tolerated by the wild type strain. In addition, the introduced *SNRK2* gene does not enable the submission strain with the ability to utilize any new or different substrates, nor does it impart any invasive properties.

Furthermore, the traits in PACE_Cs1412_SNRK2 are not new to the genus since increased growth and biomass accumulation have also been attained in wild type *C. sorokiniana* by tuning various growth parameters, which was reviewed by De Francisci et al. (2018). Table 3 shows that by adjusting basic growth parameters, researchers can tune wild type *C. sorokiniana*'s growth rate, lipid content, FAME yield, and protein content.

Table 3. Characterization of *Chlorella sorokiniana* growth and biomass reported in literature.

Research focus	Growth performance (d^{-1}/g L^{-1} d^{-1})	Lipid content (%, w/w)	FAME yield (%, w/w)	Protein content (%, w/w)	Reference
Effect of temperature	-	~10%	1.3–6.1%	-	*Patterson, 1970*
Effect of C/N ratio	-	13–46%	2.1–7.3%	-	*Chen and Johns, 1991*
Pigment composition	5.76 d^{-1}	10.00%	-	68.50%	*Matsukawa et al., 2000*
Effect of biochemical stimulants	42 mg L^{-1} d^{-1}	5–7%	-	45–60%	*Hunt et al., 2010*
Mixotrophic growth	0.44 d^{-1}	20–50%	-	10–32%	*Wan et al., 2011*
Effect of inoculum size	0.89 d^{-1}	-	-		*Lu et al., 2012*
Photoautotrophic/ heterotrophic growth	-	21–26% (P)	0.6–0.8% (P)	12–13% (P)	*Wan et al., 2012*
		20–56% (H)	12–33.6% (H)	6.2–13% (H)	
Cultivation with deep sea water	176.6 mg L^{-1} d^{-1}	51.70%	47.51%	-	*Chen et al., 2013*
Cultivation in cattle manure	12.77 mg L^{-1} d^{-1}	25–35%	12%	34%	*Kobayashi et al., 2013*
Fed-batch cultivation	3.29 d^{-1}	14.5–38.7%	12.8–34.1%	-	*Zheng et al., 2013*
Photoautotrophic/ heterotrophic/ mixotrophic growth	0.68 d^{-1} (P)	-	9.0% (P)	-	*Li et al., 2014*
	2.07 d^{-1} (H)		6.2–17.6% (H)		
	3.40 d^{-1} (M)		13.4–34.7% (M)		
Cultivation in domestic wastewater	220 mg L^{-1} d^{-1}	48.31%	-	-	*Ramanna et al., 2014*
Mixotrophic growth	1.602 d^{-1}	20–27%	-	-	*Juntila et al., 2015*
Effect of nitrogen limitation	3.21 d^{-1}	20–51%	-	-	*Li et al., 2015*
Continuous cultivation	2.41 d^{-1}, 1.52 g L^{-1} d^{-1}	-	6.24%	38.80%	*De Francisci et al., 2018*

Note: P, photoautotrophic; H, heterotrophic; M, mixotrophic.

(modified from De Francisci et al., 2018)

Although there are uncertainties associated with the small-scale field testing of the PACE_Cs1412_SNRK2, the ecological hazards associated with these small-scale tests are likely low. The introduced gene and subsequent traits does not pose unreasonable concerns for adverse ecological effects when grown within a small-scale field test environment. Since bodies of freshwater are usually inhabited simultaneously by dozens of different algal species including cyanobacteria and various green algae and other algae, it would be unlikely that adverse environmental effects would be realized even if the submission strain was to establish in local freshwater bodies near the field test site which may be likely. The only circumstances in which it seems that adverse environmental effects could be realized would be if the intergeneric strain was capable of outcompeting all other algae in the water body. As previously mentioned, three green algae, *Chlorella, Chlamydomonas,* and *Scenedesmus* are fairly ubiquitous in freshwater bodies. Therefore, there are likely to be members of these genera in all surrounding freshwater bodies. The potential ecological hazards imparted by PACE_Cs1412_SNRK2 are also considered low since the same traits can be attained by varying growth parameters of the wild type recipient (see Table 3), which can already occur naturally as environmental conditions change.

As summarized in the Ecological Hazard Assessment (Nguyen, 2018b), experiments conducted in outdoor miniponds by Szyjka et al. (2017) found that their wildtype and GE strains of the green alga *Acutodesmus dimorphus* were able to travel in bioaerosols to the maximum distance measured, 50 meters. However, the GE strains did not out-compete indigenous algae or other airborne algae taxa found in the trap buckets. In laboratory experiments conducted using local eutrophic and oligotrophic lake water containing local taxa the wildtype and GE algal strains had no measurable impact on the diversity or composition of native algae from five lakes under their experimental conditions during the timeframe of the experiment. None of the strains was able to outcompete local or airborne algae taxa in either the trap buckets or in laboratory experiments. It was also found that the GE and wildtype algae behaved in a similar manner.

Similar results are expected in these small-scale field tests where the *Chlorella* wildtype and GE strains are likely to be dispersed to other water bodies and to desert soils as *Chlorella* is known to be an "aero-algae". However, there is no reason to suspect that the GE strain would be invasive in the environmental media into which it may be disseminated. It is expected that the submission strain would be transported to other areas in the surrounding environment in bioaerosols, including potentially long distances. It is also possible that the strain could be transmitted to other environments by insects and reptiles that can access the miniponds. The submitter has agreed to the use of bird netting over the miniponds or minipond array to prevent the potential long-distance dispersal of the alga by migratory birds that are able to transport algae externally on their feet and feathers, and internally in their bills, gullets, or intestinal tracts for a hundred miles or more (Proctor, 1959).

The introduced genetic material, the *SNRK2* gene, does not pose concerns for increased pathogenicity/chlorellosis in terrestrial mammals. The introduced genetic material does not pose any toxicity concerns. The zeocin resistance encoded by the Sh *ble* gene does not pose concerns for the loss of therapeutic value of the antibiotic in animal medicine as it is a laboratory chemical that is not used to treat infections in animals.

Although there is some uncertainty regarding the dispersal of the submission strain and then its ability to establish in environments into which is disseminated, there is likely low ecological hazard associated with the small-scale field testing of PACE_Cs1412_SNRK2 for 60 days. The proposed tests to be conducted under this TERA will inform future assessments of this alga which in the future may be

genetically modified for useful products such as biofuels or biochemicals.

XIII. POTENTIAL SURVIVAL OF THE SUBMISSION MICROORGANISM

The potential for the submission microorganism to survive in water bodies or soils into which it may be dispersed has been evaluated by in the Ecological Hazard Assessment (Nguyen, 2018b). No laboratory studies to assess the potential for the algal strain to survive in natural waters have been conducted. However, the wild type and submission strains are expected to survive in fresh or marine water bodies and soils into which the strains are dispersed.

As mentioned previously, *Chlorella* is one of the three most dominant green algae in many aquatic habitats and can be frequently isolated from marine, fresh water, soils and air samples, as they can tolerate a wide range of environmental conditions (Trainor, 1998). *Chlorella* is also a simple airborne microalga, present in terrestrial and aquatic habitats, whose minute cell size and resistance against environmental stress allows for long-distance dispersal (Hodac et al., 2016). *Chlorella* is an aerophilous algae (found in air), a type of algae shown to have better adaptation and growth responses compared to their solely soil and aquatic counterparts (Sharma et al., 2007). *Chlorella* are distributed worldwide in freshwater ponds, lakes, streams, and rivers, and even in brackish waters. They are found from the arctic to the tropics. *C. sorokiniana* can survive in high temperature environments. One strain of *C. sorokiniana* was reported to have an optimal growth at temperatures between 38-42˚C (Kessler, 1985) while others report that it can be grow at temperatures ranging from 14-38°C (Patterson, 1970). Thus, *C. sorokiniana* is capable of surviving temperatures encountered in hot deserts.

Tiffany (1951), defined algae into nine different groups based on preferred habitat including edapophytes (soil algae), aerophytes (aerial algae), endophytes (living within plant tissue), and endozoophytes (living inside animal hosts), all of which are habitats in which different *Chlorella* species have been known to thrive. Lists of soil algae have been compiled across the country and the world, showing their diverse distribution, and frequently include *Chlorella* (Metting, 1981). Soil bound *Chlorella* species appear to tolerate higher levels of radiation than other more complex terrestrial life forms (Metting, 1981). Trainor (1962) was able to show that *Chlorella* is able to survive desiccation for one hour at 130°C.

As discussed in the previous section, the survival characteristics are not expected change from the wild type recipient to the submission strain. The introduced genetic material does not enable PACE_Cs1412_SNRK2 to survive in environments not tolerated by the wild type strain. The addition of the *SNRK2* gene does not enable the submission strain with the ability to utilize any new or different substrates, nor does it impart any invasive properties that did not already exist in the wild type recipient strain. Furthermore, the traits in PACE_Cs1412_SNRK2 are not new to the genus since increased growth and biomass accumulation have also been attained in wild type *C. sorokiniana* by tuning various growth parameters (Table 3) (De Francisci et al., 2018). Also, since other green algae (e.g., *Chlorella, Chlamydomonas,* and *Scenedesmus*) are fairly ubiquitous in freshwater bodies, it is unlikely that the submission microorganism would out-compete indigenous populations. Therefore, the increased growth rate and biomass accumulation provided by SNRK2 in PACE_Cs1412_SNRK2 only poses a low level of concern.

XIV. DESCRIPTION OF THE FIELD TEST SITE

The field testing of PACE_Cs1412_SNRK2 will be carried out at ASU's AzCATI Testbed facility located at 7418 Innovation Way South, Mesa, Arizona 85212. A schematic of the field test location is presented in Figure 5. The AzCATI site is located across the street from the ISTB-3 laboratory building on the Polytechnic Campus of ASU (Figure 6). It occupies approximately 4.0 acres of which 0.6 acres contains production facilities with a cultivation capacity of 300,000 L. The site includes 3 greenhouses and 2 head-houses and is under the direct control and supervision of AzCATI personnel. The site is fenced-in with restricted access limited to authorized personnel. The site contains 32 miniponds (0.5-5 m^2, and volumes of 0.1-1.5 m^3), 2 medium raceways (60 m^2, volume up to 15 m^3), and ARID™ raceway (400 m^2, volume up to 40 m^3), and 1 large raceway (500 m^2, volume up to 125 m^3). In addition, the site has 50+ closed photobioreactors in different configurations (flat panel, hanging bag, plastic/glass tubular) in volumes from 0.025-1.5 m^3).

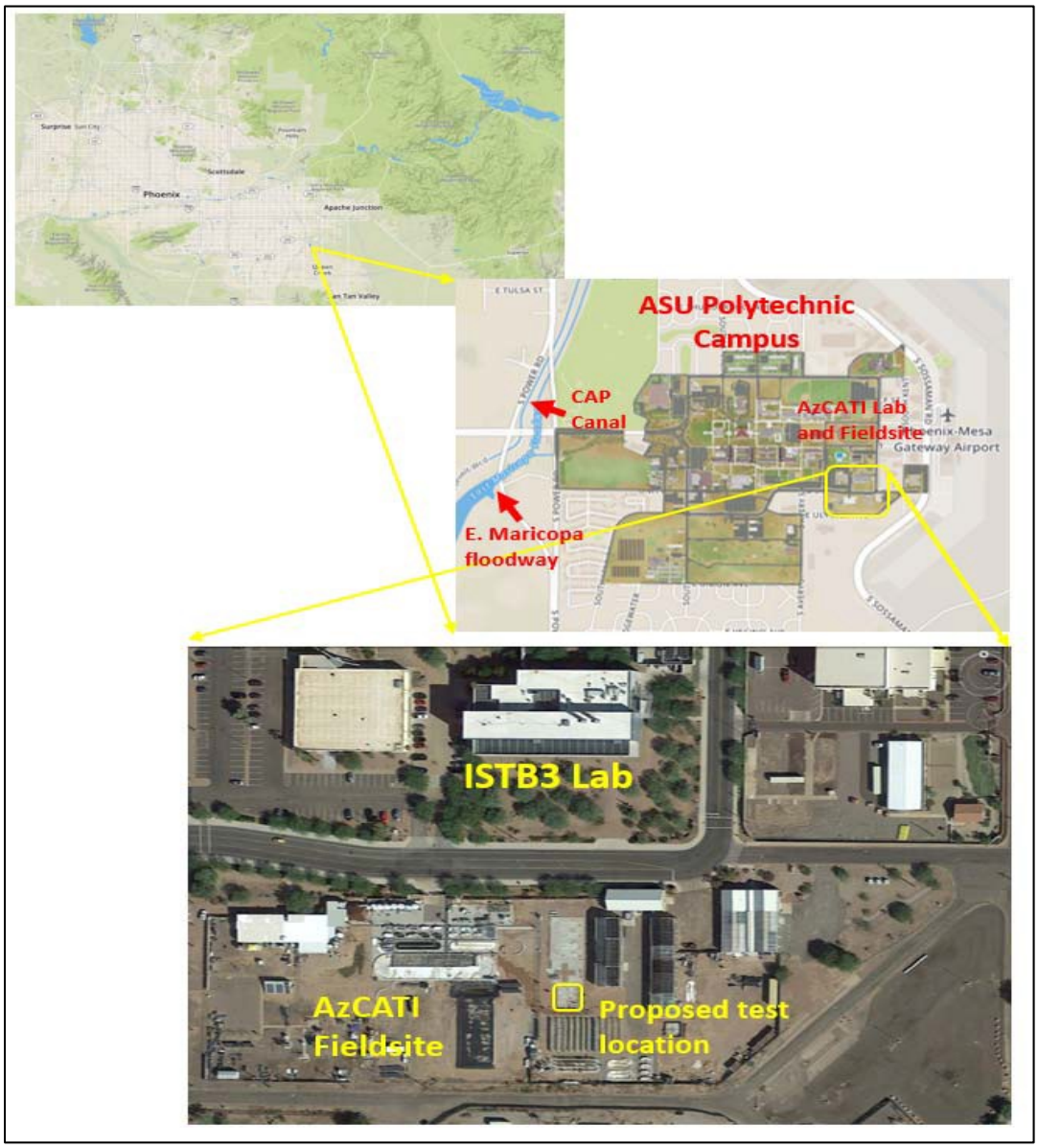

Figure 5. Location of the AzCATI site on the ASU Polytechnic Campus.

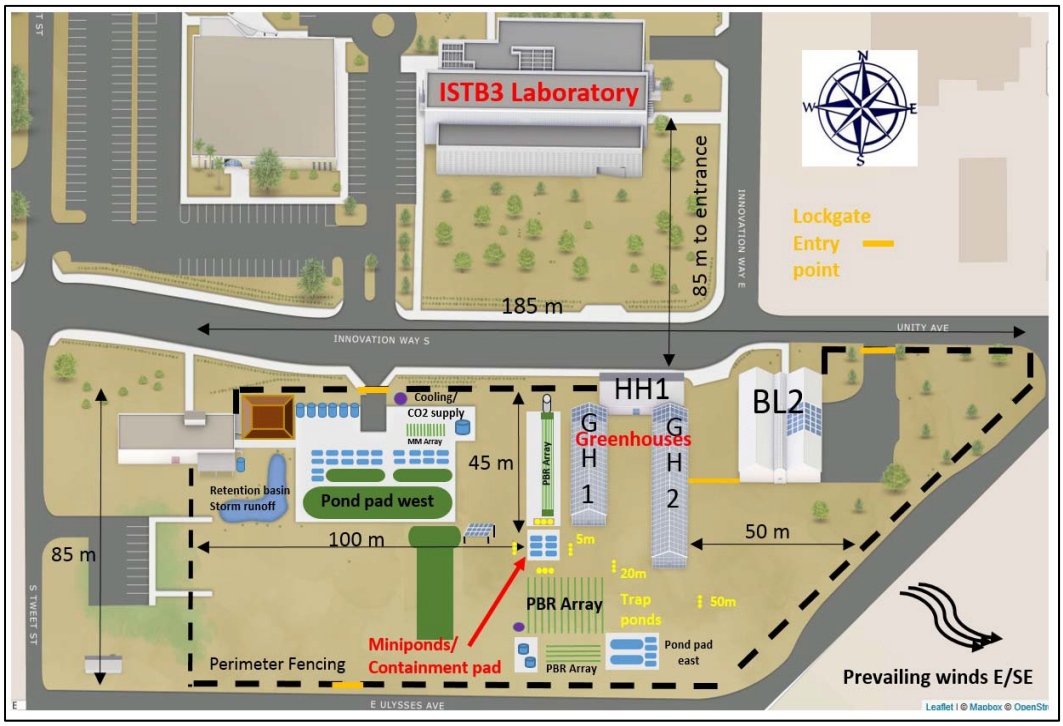

Figure 6. Layout of the AzCATI field testing facility

The area within AzCATI that will be used to cultivate the submission microorganism is a minipond array of 6 raceway miniponds, sitting in a 9 m x 11 m containment area with surrounding trap ponds placed along the AzCATI Testbed. This site was built for the purpose of performing algae cultivation trials at various scales outdoors. There has been active outdoor cultivation work since 2006, with major expansions in 2008 and again in 2011. It is co-located on the polytechnic campus of Arizona State University and immediately adjacent to a 30-square foot state-of-the-art laboratory facility. The submitter has 10 years of experience of working with naturally occurring and genetically engineered algae and cyanobacteria under closed cultivation conditions, and outdoor cultivation of naturally occurring strains of algae including at scales >1,000 L. This site also serves as the lead site for a Department of Energy funded national testbed network, the Algae Testbed Public-Private Partnership (ATP^3), which has separate funding than that for the PACE consortium.

In the Phoenix area, water comes from one of three sources: groundwater (either from a private well or one operated by a city or private water company), the Salt and Verde rivers (delivered by Salt River Project- SRP) or the Colorado River (brought here by the Central Arizona Project – CAP) in a canal that stretches 336 miles from Lake Havasu to Tucson. The site is not within close proximity to any natural lakes. The two largest reservoirs are Roosevelt Lake (70 km to the northeast) and Lake Pleasant (80 km to the northwest). There is a CAP/SRP water canal that runs along the west side of the campus approximately 1.5 km due west of the field site. The soil type across the campus and in particular on the field site is of a hard-packed caliche type (i.e., soil that is cemented together by calcium carbonate) that is alkaline and is very common in southern Arizona.

The size of the ponds to be used for cultivation of the submission strain are called “miniponds” with volumes between 800 – 1,000 L, and they sit within a 9 m x 11 m containment pad, along with the tank disposal/harvest area (Figure 7). The submitter states that bird netting will be added either over

individual ponds (likely) or over entire area.

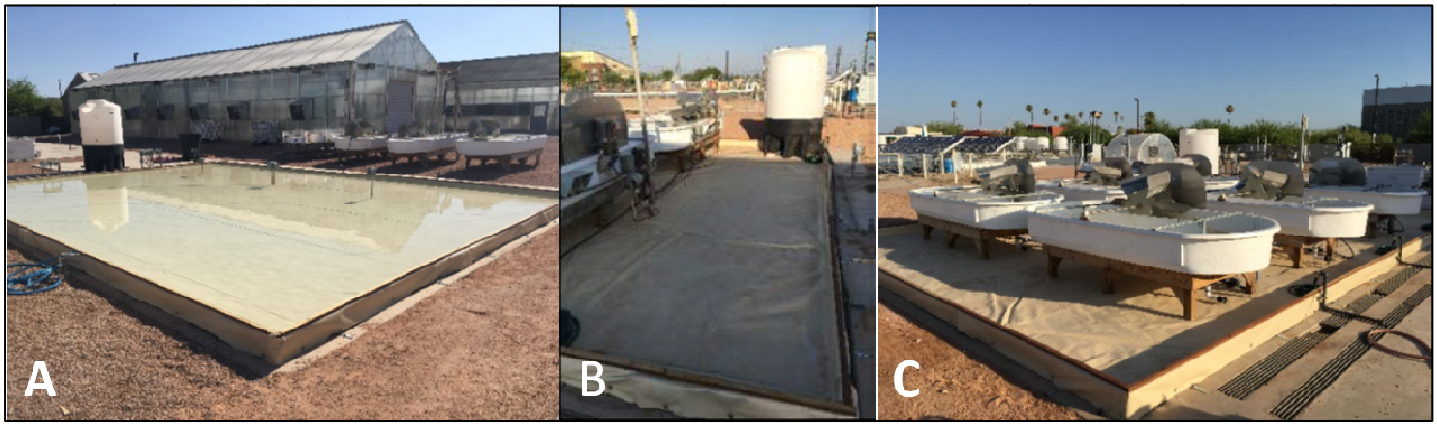

Figure 7. AzCATI GM containment pad for GM algae testing (R-18-0001). A) Containment pad (9 m x 11 m) leak test. B) Disposal/harvest tank area. C) Minipond array of 6 raceway miniponds (holds 800 - 1,000 L each, with paddle wheels for circulation) sitting in the containment pad.

XV. STUDIES TO BE CONDUCTED TESTS AT THE FIELD TEST SITE

The purpose of this field trial is (1) to evaluate the translatability of GM phenotypes from a lab to an outdoor setting, and (2) to compare the submission and recipient microorganisms when cultivated in outdoor miniponds with respect to their ability to produce biomass, and (3) also to identify any increase or decrease in biomass productivity under biotic (bacteria, predators) and abiotic (diurnal temp and light) stressors from the environment.

Throughout scale up and the outdoor trial, the submitters will confirm at each transfer stage that they have the correct submission and recipient microorganism through PCR with specific primers developed allowing discrimination from other *Chlorella* sp., including the recipient, as well as of course other algal species. GM algae are detectable *in situ* by unique intrinsic DNA sequences/and or inserted genetic markers (Henley et al., 2013). Parameters to be measured routinely for growth analyses are presented in Table 4.

For the second objective of characterizing the dispersal and invasiveness of the submission microorganism in the local ecosystem, the submitters will employ water traps external of the miniponds as means to assess the dispersal capability by gathering algae samples that escape the mass cultivation. Captured algal cells will be concentrated through centrifugation or filtration of the media/water sample. PCR amplification of the DNA extracted from the concentrated cell sample will indicate the presence or absence of the GM algae using the PCR amplification procedure outlined in Liu et al. (2014) with primers specific to the inserted transgenes. Prior to mass cultivation, a qPCR experiment will establish the minimum detection limit of the marker of interest for the submission microorganism to establish the minimum number of organisms required to see a positive result in the screening. With this calibration, they will be able to monitor the trap ponds as well as other cultivation systems that will be running on the field site and be able to back calculate the number of cells/ml. Miniponds, trap ponds and other

AzCATI field site ponds will be running on the field site and the submitters will be able to back calculate the number of cells/ml. Miniponds, trap ponds, and other AzCATI field site ponds will be sampled at the rates shown in Table 4. For the field testing, at least 6 and up to 12 trap ponds will be used for the dispersion testing. These will contain either water from a surface source or synthetic media matching what is used on the proposed cultivation trials.

Table 4. Parameters to be measured for algae growth analyses and weather conditions.

Measurment	Grab Sample Amount (if applicable)	Frequency of Sampling	Place of Sampling	Storage Conditions
Pond pH/Temperature	N/A	daily	containment miniponds	N/A
$OD_{750/680}$	100 ml (same grab sample as AFDW)	daily	containment miniponds	N/A
DW/AFDW	100 ml (same grab sample as OD)	daily	containment miniponds	N/A
Nutrient Levels in Media (N:P)	5 mL (Supernatunt from OD/AFDW)	daily	containment miniponds	N/A
Microscopic Observation (culture health)	5 ml (same grab sample as OD/AFDW)	daily	containment miniponds	N/A
Pulse Amplitude Modulation (PAM: Photosynthetic efficiency)	10 ml (liquid)	3x/week	containment miniponds	N/A
Pond samples (qPCR)	10 ml (pellet)	daily	containment miniponds	-20°C
Proximate Analysis (total FAME, Total Protein, Total Carbohydrate, ASH)	1-4 L	1-3x/week	containment miniponds	-20°C (freeze dried)
In-situ YSI 5200 sensors (pH, pond water temperature, salinity, % oxygen saturation)	N/A	15 minutes	containment miniponds	N/A
Environemntal (RH, Air Temp, wind speed, wind direction, total irradiance (W/m^2), PAR	N/A	Hourly	weather station	N/A
Precipitation	N/A	Hourly	PHX/Gateway Airport	N/A
Trap pond samples (qPCR)	10 ml (pellet)	3x/week	Trap Ponds	-20°C
AzCATI pond/pbr samples (qPCR)	10 ml (pellet)	weekly	AzCATI ponds/PBRs	-20°C
Bulk harvest (HTL)	500-800 L	1x every 1-2 weeks	containment miniponds	-20°C (freeze dried)

XVI. EXPOSURE ASSESSMENT

For a detailed account of potential releases of the production microorganism during laboratory propagation, growth, and waste disposal, see the Engineering Report (Macek, 2018).

1. Production Volume

The submission indicates that the miniponds will be run semi-continuously between 800-1,000 L at a density ranging from 0.1 to 1.0 g/L. The miniponds will be inoculated between 0.1 to 0.2 g/L and biomass will be harvested from the miniponds when the density reaches between 0.4 to 1 g/L. The submitter expects to harvest 50-80% of biomass in the miniponds between 1 to 10 times during the

experiment at a maximal density of 1.0 g/L. Each harvest may take place between 5 and 15 days after inoculation. The submitter expects to utilize six miniponds. As a conservative estimate, 6 miniponds and 10 harvests per minipond were assumed. The technical contact indicated a harvest density of 0.2 to 0.5 g/L (~5 to 12 x 10^7 colony-forming units (CFU)/mL). Thus, the total maximum production volume for the submission microorganism PACE_Cs1412_SNRK2 is 7.2 x 10^{15} CFU for this 60-day field trial.

2. Process Description

The submission microorganisms are received at the AzCATI laboratory under the PACE Material Transfer Agreement, and are subject to an NIH R&D exemption (40 CFR parts 725.234 and 725.235). The strain will be transported on agar plates and scaled up in closed systems.

The recipient and submission microorganisms will enter the AzCATI laboratory on agar plates and will be scaled in through small shaker flasks, 800 mL bubble columns, to 2 ft. X 2 ft. flat panel photobioreactors (PBRs) with a 2.0-inch light path operated at maximum volume of 10 L. When the appropriate density (2.5 g/L) is reached in the PBRs, they will combine culture in approved containers with secondary containment to transport seed culture from laboratory to field site (approximately 100 meters away) to be used to inoculate the miniponds. The miniponds will be inoculated to a starting density of at least 0.1 g/L at a depth of 800-1,000 L and 4.2 m^2 in surface area (entry weight of 0.24-0.3 kg biomass per minipond). The miniponds will be cultivated, samples will be taken, and biomass will be harvested when the culture reaches a density of 0.4 g/L to 1.0 g/L ash free dry weight (AFDW). Samples will be inactivated by treatment with 5% bleach solution and autoclaved. Bulk cultures will also be inactivated by treatment with bleach and disposed into the sanitary sewer (per large scale culture disposal protocol).

Samples will be taken to perform several analyses and *in situ* parameters will be monitored (Table 3). Daily readings of pH and temperature will be performed. *In situ* YSI 5200 sensors measure pH, pond water temperature, salinity, and percent oxygen saturation in the miniponds every 15 minutes. Grab samples will be taken daily for measuring $OD_{750/680}$, dry weight (DW)/ash-free dry weight (AFDW), nutrient levels in media (N:P), and qPCR. Samples will be taken three times a week for Pulse Amplitude Modulation (PAM) measurements. Grab samples (1-4 L) will be taken one to three times per week for proximate analysis (total fatty acid methyl ester (FAME), total protein, total carbohydrate, and ash). Bulk harvest of the miniponds (500-800 L) will occur once every 1-2 weeks. All field test samples will be inactivated with 5% bleach or autoclaving before disposal via the sewage system and samples for future analyses (proximate analysis, qPCR, bulk harvest) will be freeze-dried at -20°C which renders the cells non-viable.

Samples that are transported from laboratory and field site will be labeled with designated information including, batch ID, source location, date, strain ID, collectors name and purpose of the sample. Samples that are transported between laboratory and field site will be placed into double containment, so that spills/leaks do not occur.

Harvest will be triggered by biomass density 0.4 g/L to 1.0 g/L AFDW and the harvested volume will be calculated to target a new dry weight of 0.4 g/L once the harvested volume is replaced by new media. The harvested material will be dewatered to a slurry via centrifugation and the concentrated algae paste will be freeze dried or stored as frozen (at -20°C). Remaining biomass from the centrifugation will be dosed with 5% bleach or autoclaved before disposal.

Once the field experiment has been terminated, all biomass will be inactivated by bleaching or autoclaving. All equipment will be cleansed of the submission microorganism (including all sample containers, ponds, etc.) by bleaching, autoclaving and will be discarded as necessary. Any pond spill will be contained within the containment area, flooded with 5% bleach solution, allowed to soak overnight, and liquid then disposed of in the sewage system.

3. Worker Exposure

The occupational exposure to the five algal submission strains for the proposed field test has been estimated by Macek (2018). There will be a total of up to 10 workers (all activities except sample processing).

The worker estimates provided in the submission are given in Table 5.

Table 5. Worker estimates by activity.

Worker Activity	PPE	# of Workers Exposed	Maximum Duration (hrs/day)	Maximum Duration (days/yr)
Initial Application (scale-up and inoculation)	Gloves, lab glasses, lab coats, long pants, and closed-toe shoes.	2	2	30
Routine pond monitoring (pH, temperature)		2	2	60
Sampling of ponds (scope observations, OD, AFDW, qPCR, etc.)		2	2	60
Sample processing		3	6	45
Pond harvesting		2	6	10
Experimental termination		2	4	3

The submission indicates that proper personal protective equipment will be worn by all on-site staff as required by ASU EH&S regulations.

INHALATION EXPOSURE (bioaerosols):

1) From Pond Harvesting: 360 to 2500 CFU/day, up to 8 workers (initial application, pond monitoring, sampling, and pond harvesting), 10 days/yr
2) From Sample Processing: negligible

DERMAL EXPOSURE:

1) From Daily Sample Processing: 4.8×10^7 to 1.3×10^8 CFU/day, up to 5 workers (sample processing and experimental termination), 60 days
2) From Loading of Algae Paste into Bottles: 9.0×10^7 to 2.6×10^8 CFU/day, 10days/yr

This dermal exposure estimate assumes spillage onto unprotected skin. However, PPE (i.e., gloves) will be worn.

4. Environmental Releases

WATER:

1) From Equipment Cleaning: 1.4 x 10^{11} CFU/day over 10 days/yr; 1.4 x 10^{12} CFU/yr
2) From Centrifuge Supernatant Waste: 7.2 x 10^{10} CFU/day over 10 days/yr; 7.2 x 10^{11} CFU/yr
3) From Paste Disposal: 7.0 x 10^{12} CFU/day over 10 days/yr; 7.0 x 10^{13} CFU/yr
4) From Sample Wastes: Negligible

AIR:

1) From Bioaerosol Emissions: 1.2 x 10^{5} CFU/day over 60 days/yr; 7.2 x 10^{6} CFU/yr
2) From Fugitive Emissions from Centrifuge: 1.2 x 10^{5} CFU/day over 60 days/yr; 7.2 x 10^{6} CFU/yr
3) From Fugitive Emissions During Sampling: Negligible

LANDFILL: Not expected

INCINERATION: Not expected

5. Consumer, General Population, and Environmental Exposure

The exposures to consumers, the general population, and to the environment were estimated by Lynch (2018).

a. Consumer Exposure

The algal submission strain is not intended for use in consumer products. Therefore, exposure to consumers is not expected.

b. General Population Exposure

There are releases of the algal submission strain to air and wastewater treatment from processing. However, wastewater management practices and low emissions to air are expected to result in negligible exposure levels (Lynch, 2018).

Exposure to Releases from Processing

1. Inhalation Exposure from Bioaerosol Emissions

Using the estimated maximum release of 1.4 x 10^{7} CFU/yr to air, the concentration in ambient air 100 meters downwind would be < 1 CFU/m^3. Thus, exposures are expected to be negligible (< 1 cfu/yr).

2. Drinking Water Exposure from Equipment Cleaning Waste and Disposal of Cell Paste

Equipment cleaning and disposal of cell paste will occur during harvesting of the TERAs at one site over 10 days per year.

EPA estimated that a total of 1.4 x 10^{11} CFU/day over 10 days/yr could be discharged to water from equipment cleaning, 7.2 x 10^{10} CFU/day over 10 days per year could be discharged to water from centrifuge supernatant, and 7 x 10^{12} CFU/day over 10 days/yr could be discharged to water from paste disposal (Macek, 2018). Aqueous wastes from the processing site are discharged via sewer to the City of Mesa's Greenfield Water Reclamation Plant. According to the Town of Queen Creek website, the facility

treats approximately 16 million gallons per day of wastewater by activated sludge with nitrification/denitrification treatment. After it is treated to secondary effluent standards, Mesa's portion of the treated effluent is sent to the Gila River Indian Community as part of a water exchange agreement. The effluent is managed under the Wastewater and Reclaimed Water Management ordinance of the Gila River Indian Community.

The ordinance prohibits direct human consumption of, use in food preparation, or for drinking or swimming or other full or partial immersion activities with potential for ingestion. Point source discharge of reclaimed water for indirect potable groundwater recharge may be allowed by permit pursuant to the criteria of the Safe Drinking Water Act. Agricultural reuse may be permitted with approval by the Gila River Indian Community Department of Environmental Quality.

The treated biosolids are approved for use in non-edible land application for agriculture. The effluent is not discharged directly to any receiving stream.

If it is assumed that the submission strain is not removed during the wastewater treatment process and that cleaning wastes and cell paste waste is released and treated simultaneously, their concentrations in the effluent of the Greenfield Water Reclamation Plant would not be expected to be greater than:

7.21×10^{12} CFU/day / 60.6 MLD Plant Flow = 1.19×10^{5} CFU/L.

It should be noted that treated effluent is not released directly to surface waters. Because there are no direct releases of water containing the submission strain to drinking water sources, the potential for general population exposure to the submission strain via drinking water is eliminated. Factors including removal of the submission strain in the Greenfield wastewater treatment plant, survival in soil subsurface and groundwater environments, and removal in drinking water treatment are expected to further mitigate exposure via drinking water ingestion. However, if treated Greenfield effluent is used for indirect potable groundwater recharge, the potential presence of low concentrations of the submission strain in groundwater used as drinking source cannot be ruled out.

XVII. INTEGRATED RISK ASSESSMENT

Although there are some uncertainties regarding the ability of the submission strain PACE_Cs1412_SNRK2 to be dispersed from the testing site through aerosols and the subsequent survival of the alga in the environmental media into which it may be disseminated, the proposed small-scale field test does not appear to pose unreasonable risks to human health or the environment.

The recipient alga, *Chlorella sorokiniana,* is not known to be pathogenic to humans. Although there have been three cases of infection in humans caused by unspecified species of *Chlorella,* chlorellosis in humans is extremely rare as *Chlorella* is omnipresent in the environment in both fresh and marine waters, and in soils so humans are frequently exposed to the alga. Also, these three infections were a result of open wounds being exposed to contaminated waters. The introduced SNRK2 enzyme in the submission strain does not pose any increased concern for human pathogenicity. It is a common enzyme found in many organisms including humans. The introduced genetic material does not pose increased risk of pathogenicity to susceptible subpopulations, i.e., immunosuppressed individuals. Even in extremely rare circumstances of exposure of open wounds in severely immunocompromised individuals to contaminated waters, the submission strain is not expected to pose any concerns that are not already

associated with the wild type recipient strain.

The use of the antibiotic resistance marker gene, *ble*, that encodes resistance to zeocin also does not pose human health concerns. Zeocin is a laboratory chemical not approved for use in human or animal medicine. Thus, there is not the usual concern for the potential loss of therapeutic value of an antibiotic if there was horizontal gene transfer of the antibiotic resistance gene to pathogens in the environment whose infections are treated with that antibiotic.

There is no concern for toxicity to humans because no species of *Chlorella* is known to produce phycotoxins. *Chlorella* sp. are commonly used as human dietary supplements, and thus, do not pose toxicity concerns. The introduced genetic material does not pose any toxicity concerns even to susceptible subpopulations.

There is low concern for allergenicity in workers and to the general population from exposures to the genetically modified algae during this field test. Naturally occurring strains of *Chlorella* have been grown outdoors at the AzCATI facility for 10 years with no reports of allergy symptoms in the workers. The frequent detection of *Chlorella* in bioaerosols in the environment implies that humans are routinely exposed to *Chlorella* by the respiratory route. Adverse allergic reactions to algae in bioaerosols is thought to be less of a concern that other airborne environmental antigens such as bacteria, fungi, and pollen spores.

However, *Chlorella,* and thus the submission strain, may cause sensitization in the susceptible subpopulation of atopic individuals, i.e., those with a genetic predisposition toward developing hypersensitivity reactions upon exposure to environmental antigens. *Chlorella* is suspected of being a weak allergen although no studies have established a definite causal role for *Chlorella* in human respiratory allergies. The algal cells are expected to occur in bioaerosols generated through the turbulence of the paddle wheels in the raceway miniponds or through wind action. Since *Chlorella* cells are very small and growth is unicellular, the alga is known to be easily transported in the air. It is unlikely that atopic individuals would choose to work with algae at the AzCATI test bed facility given their predisposition for respiratory hypersensitivity reactions with exposure to environmental antigens. However, if any workers are atopic, or non-atopic individuals were to developed allergy symptoms, the use of respirators would mitigate allergenic responses.

There is no increased concern for the respiratory exposure of the submission microorganism PACE_Cs1412_SNRK2 compared to that of the recipient alga. The SNRK2 enzyme is intracellular, thus, there is no direct respiratory exposure to the protein. The zeocin resistance gene product did not show high sequence similarity to known allergens. There is low concern for allergenicity through respiratory exposure of the submission strain PACE_Cs1412_SNRK2 to the general population as inhalation exposures resulting from this small-scale field test are expected to be quite low.

Likewise, environmental hazards resulting from this small-scale field test are expected to be low. Although there have been a few cases of chlorellosis in several animals, infection of non-human mammals by *Chlorella* is very rare as there are a limited number of cases even though *Chlorella* is one of the most prevalent algae in the environment in marine and fresh waters and in soils. There is low concern for potential toxicity of *Chlorella* to animals since no members of the genus are known to produce phycotoxins. There is no literature suggesting that *Chlorella* has any adverse effects on terrestrial or aquatic plants.

As previously discussed in the Ecological Hazard section, the genetic modifications presented for this submission enhances the growth and biomass accumulation of the submission microorganism, which can be viewed as an increased competitive advantage in the environment as it will consume more nutrients at a faster rate than that of the wild type recipient. However, the survival characteristics are not expected to drastically change from the wild type recipient to the submission strain. The introduced genetic material does not enable PACE_Cs1412_SNRK2 to survive in environments not tolerated by the wild type strain. The addition of the *SNRK2* gene does not enable the submission strain the ability to utilize any new or different substrates, nor does it impart any invasive properties. Furthermore, the traits in PACE_Cs1412_SNRK2 are not new to the genus since increased growth and biomass accumulation have also been attained in wild type *C. sorokiniana* by tuning various growth parameters (Table 3) (De Francisci et al., 2018).

As discussed by Henley et al. (2013) in their analysis of the risks posed by commercial scale production of GE algae, there are several scenarios that should be considered if a GE alga is grown outdoors, and hence, are likely to be disseminated to other environments. It is possible that a GE algal strain would die off in a new environment, and thus, there would be low risk. However, even the scenario of low level survival of a GE strain in the environment does not in and of itself pose risk. If there is low level survival of the GE strain, then the selective advantage imparted to indigenous populations through horizontal gene transfer must be considered. If the horizontally transferred trait imparts a nonsignificant selective advantage to indigenous species, then there is low risk. If the horizontally transferred trait imparts a significant selective advantage to indigenous species, then there could be some risk. According to the authors, the scenario of high risk is when the GE algae dominates a new environment and causes hazardous algal blooms or ecosystem-disruptive algae blooms (EDABs). As previously discussed, *Chlorella* do not produce phycotoxins and thus do not cause HABs.

Hence, the only scenario that would present an unreasonable risk is if the submission strain, PACE_Cs1412_SNRK2 was to outcompete indigenous algae in the environment and disrupt the new ecosystems. It may also be important to consider if any perturbations in an ecosystem are temporally limited or persistent. As previously discussed in the Ecological Hazard section, since bodies of freshwater are usually inhabited simultaneously by dozens of different algal species including cyanobacteria and various green algae and other algae, it would be unlikely that adverse environmental effects would be realized even if the submission strain was to establish in local freshwater bodies near the field test site which may be likely. As previously mentioned, three green algae, *Chlorella, Chlamydomonas,* and *Scenedesmus* are fairly ubiquitous in freshwater bodies. Therefore, there are likely to be members of these genera in all surrounding freshwater bodies. None of the introduced genetic material impart invasiveness characteristics to the submission strain.

The potential for horizontal gene transfer of the *SNRK2* gene to other algae in the environment is thought to be low as *Chlorella* is not known to readily exchange genetic material horizontally. Very little is known about horizontal gene transfer in eukaryotic algae as it has not been observed. Vertical transfer of the introduced genetic material through sexual reproduction to other *Chlorella* species is also expected to be low. Although the researchers have reported that sexual reproduction of *Chlorella sorokiniana* is possible, inducement of gametogenesis is required in the laboratory and is not expected to occur naturally. More specifically, the recipient strain *C. sorokiniana DOE1412* has not been reported anywhere to be capable of sexual reproduction.

Even if the introduced *SNRK2* gene allows the submission strain to grow faster and accumulate more biomass, there is low concern for it out-competing indigenous strains in different environments since

the addition of the *SNRK2* gene does not enable the submission strain the ability to utilize any new or different substrates, nor does it impart any invasive properties that did not already exist in the wild type recipient strain. Thus, even though the submission strain may be dispersed into other environments as is the case with any algae grown outdoors, there is low risk associated with the dispersal to and survival in other environments into which it may be disseminated.

CONCLUSIONS

Although there are some uncertainties regarding the ability of the submission strain, PACE_Cs1412_SNRK2 to survive in environments into which it may be dispersed, the proposed field test does not appear to present an unreasonable risk to human health or the environment. This small-scale field test is necessary to test the outdoor growth of the algal submission strain, and to generate data regarding dispersal and competition with native populations in waters collected from the surrounding environment. These data will inform future assessments of this alga, which depending on the outcome of this small-scale field test, may be further genetically modified for useful products such as biofuels or other bioproducts.

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